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AGE- AND PREGNANCY-RELATED CHANGES IN SERUM TOTAL CHOLESTEROL AND TRIGLYCERIDE LEVELS IN THE CAYO SANTIAGO RHESUS MACAQUES

MATT J. KESSLER* and RICHARD G. RAWLINS**

*Caribbean Primate Research Center, University of Puerto Rico, School of Medicine, P.O. Box 1053, Sabana Seca, PR 00749, and **Endocrine Research Unit, Michigan State University, East Lansing, MI 48824

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INTRODUCTION

A RECENT REPORT on lipid levels in a small group of individually caged rhesus monkeys (Szymanski and Kritchevsky, 1980) indicated that serum total cholesterol and triglyceride values were higher in older monkeys. Our study was intended to extend the findings of that report to a large group (n = 96) of provisioned free-ranging rhesus monkeys of known age and lineage on the island of Cayo Santiago, Puerto Rico. Significant differences were found between cholesterol levels in juvenile and adult pregnant females and between adult pregnant females and adult males. Cholesterol levels decreased in pregnancy and increased with age in males. Triglyceride values were significantly lower in adult and aged males than in juvenile males. The results of this report indicate that cholesterol levels in male monkeys tend to increase with advancing age; that cholesterol levels decrease during pregnancy; and that triglyceride levels tend to decrease with age in male rhesus monkeys.

SUBJECTS AND METHODS

The rhesus monkeys (*Macaca mulatta*) of Cayo Santiago, Puerto Rico, represent a unique research resource. The 792 animals resident on this 15.2 ha. island are descendants of the original group of monkeys trapped in India and transported to the island in 1938–1939. No new monkeys have been introduced into the colony since its inception. The monkeys here have been the subjects of intensive behavioral and anthropological study over the past 24 years yielding detailed information on their genealogies, behavior, dominance, diseases, and reproduction. They probably represent the most homogeneous and intensively-studied free-ranging population of rhesus monkeys anywhere in the world.

The subjects of this study were the members of social group L, one of six naturally-formed troops on the island. Figure 1 presents a graphic description of the sex and age distribution of the 96 monkeys in this troop which were sampled for this study. The monkeys were arbitrarily divided into 3 study groups: juveniles (2–3 years of age); adults (4–13 years of age); and, aged (15–26 years of age). All adult females (n = 22) sampled

Correspondence and requests for reprints should be addressed to Dr. Matt J. Kessler.

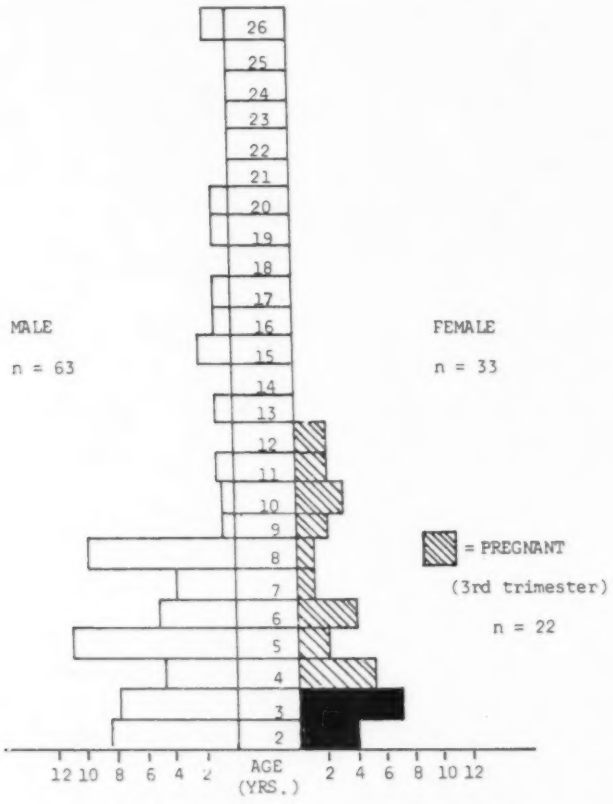

FIG. 1. Age/Sex composition of sampled population.

were in their third trimester of pregnancy. The non-gravid females represented only 8.3% of the adult females (2 of 24) in the troop, and were dropped from the study because of the small sample size. The 96 subjects consisted of the following animals: 63 total males (17 juveniles, 39 adults, and 7 aged); and, 33 total females (11 juveniles and 22 adult pregnant). All monkeys were provisioned with a high protein commercial monkey diet (Wayne or Purina) and water *ad libitum*, and had foraging access to all local vegetation, soil, insects, and small vertebrates. Each of the monkeys sampled was captured during the annual trapping session and fasted overnight. Ten minutes prior to venapuncture, the subjects were administered a restraint dosage of 10 mg/kg ketamine hydrochloride intramuscularly to facilitate handling. Venous blood samples were obtained from a femoral vein using standardized techniques. The blood was allowed to clot at room temperature and then refrigerated to retract the clot. The serum was collected after centrifugation and then sent to a commercial laboratory where the samples were analyzed using a standardized micro-technique (Chem-Screen® Profile[1]).

RESULTS

The mean serum cholesterol and triglyceride levels are presented in Table 1. Statistical comparisons were made between means using analysis of variance and between age and triglyceride values of male monkeys using linear regression. Cholesterol values were significantly lower in adult pregnant females than in juvenile females ($p < .005$), and were higher in adult and aged males than in juvenile males ($p > .1$). Triglyceride levels

[1]Metpath, Inc., Teterboro, N.J.

TABLE 1. SERUM TOTAL CHOLESTEROL AND TRICLYCERIDE LEVELS OF RHESUS MONKEYS

Serums	Juveniles (2–3 yrs.) M	F	Adults (4–13 yrs.) M	F*	Aged Adults (15–26 yrs.) M	F
Cholesterol (mg/dl)						
Mean	139.9	140.6	152.9	95.9	149.0	–
Std. Dev.	24.4	29.0	29.0	25.6	24.7	–
Number	17	11	39	22	7	–
Triglycerides (mg/dl)						
Mean	123.3	130.6	95.5	110.0	90.7	–
Std. Dev.	56.3	49.4	31.2	43.8	39.6	–
Number	17	11	39	22	7	–

*Third trimester pregnancy.

decreased with pregnancy and/or age in the females ($p < .005$) and with age in males with significant differences found between juvenile males and adult males, ($p < .05$) and juvenile males and aged males ($p < .05$). However, the linear regression statistic did not indicate a strong inverse relationship between age and triglyceride levels ($r = -.27$) in the males.

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DISCUSSION

The data indicate some significant differences associated with pregnancy in the females and with advancing age in the males. Pregnant adult females had significantly lower levels of cholesterol than juvenile females. Previous reports (Allen and Ahlgren, 1968; Martin, Wolf and Houser, 1973; Fujiwara *et al.*, 1974) on the effects of pregnancy on cholesterol levels in adult macaques have clearly shown marked depression (approximately 50%) in early pregnancy with return to normal cholesterol levels after parturition. Our findings in free-ranging rhesus monkeys are in accord with previous studies in laboratory macaques.

Adult and aged males had significantly lower triglyceride levels than juvenile males. This finding is inconsistent with age-related changes reported recently (Szymanski and Kritchevsky, 1980) in which there was a 61% higher triglyceride level in old (5–10 years) rhesus monkeys, and with other findings (Lacko and Hazzard, 1979) in *Macaca nemestrina* in whom triglycerides tend to increase with advancing age in three age-scaled groups of monkeys. We have no explanation for this apparent discrepancy.

The only significant differences between sex and age groups were found in cholesterol levels of pregnant females vs. adult or aged males. Because all adult females were pregnant and, therefore, had depressed cholesterol levels, this data represents an unrealistic comparison.

SUMMARY

Ninety-six free-ranging rhesus monkeys were evaluated for age-, sex-, and pregnancy-related changes in total serum cholesterol and triglyceride levels and compared with previous studies. Our findings indicate that pregnancy depressed total cholesterol in females and that cholesterol levels tend to increase in males with age. Triglycerides decreased significantly with advancing age in males.

The Cayo Santiago rhesus monkeys represent a unique opportunity to study the effects of age on a population of nearly 1200 nonhuman primates on which there is accurate data on birth date, lineage, behavior, reproduction and post-mortem morphology (skeletons). Further gerontological studies are necessary to take full advantage of this resource and to increase the presently-scant body of information on aging in monkeys for comparative studies on humans and for the development of animal models of gerontological diseases of humans.

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BIOLOGICAL AGE AND ITS ESTIMATION. II. ASSESSMENT OF BIOLOGICAL AGE OF ALBINO RATS BY MULTIPLE REGRESSION ANALYSIS

T.L. DUBINA*, V.A. DYUNDIKOVA*, E.V. ZHUK†

*Laboratory of Biochemistry, Sector of Gerontology, Academy of Sciences of the B.S.S.R.,
†Institute of Physiology, Academy of Sciences of the B.S.S.R., Minsk, U.S.S.R.

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Abstract—Multiple regression model of biological age (BA) theoretically gives agreement with the main concept of BA. When assessment of BA is based on the model, the age being in regression center, the method provides satisfactory results, whereas BA estimates of individuals in extreme age groups are erroneous. Investigation of male and female Wistar rats of age 5–29 months showed the BA estimates calculated from 4–10 physiological indices in young (5–7 mo) animals are overestimated, and in old (24–28 mo) animals are underestimated. Coincidence of average BA in one-age group of animals with its chronological age served as a criterion for the correspondence of the estimate to "real" BA. The paper also examines the following questions: the necessary and sufficient number of physiological indices; the sample size from the intact animal population to establish normal aging standard; the relationship between BA and animal weight.

INTRODUCTION

DETERMINATION OF biological age (BA) of laboratory animals in experimental gerontological studies is of importance as a method of estimating the efficiency of interference with aging. A standardized procedure of determining the animal BA might be of interest for other biomedical studies. In the problem two aspects can be clearly distinguished. The first is selection of adequate parameters, which characterize the age-related changes of the physiological functions and intensity of the basic aging processes. The second aspect is a choice of the mathematical tool to assess the individual's BA or BA of the groups to be compared on the basis of the measured physiological parameters.

In the previous paper (Dyundikova *et al.*, 1981) some physiological indices of male and female Wistar rats were considered in regard to their validity as BA tests. This work deals with assessment of BA of the rats by multiple regression analysis using some of the parameters measured. This mathematical approach was used for estimation of BA (in time units) of man and recognized as applicable for detection of some differences in the mode of aging of subpopulations which differ from the main populations in such features as arterial hypertension (Furukawa *et al.*, 1975) or health status (Webster and Logie, 1976). It was also shown that BA of rats calculated with the multiple regression analysis

Address all correspondence to Dr. T.L. Dubina.

can be used to evaluate the effect of three fast days repeated periodically (Dubina *et al.*, 1979). The multiple regression model of BA was resultative in the studies on the influence of moderate dietary restrictions, physical training, a combination of the two factors and the action of subcutaneously applied testis material on aging of rats (Skalicky *et al.*, 1980).

In all the above works BA of man subpopulations studied or BA of the treated animal groups were calculated from comparison with the normal populations as "a standard of normal aging" (Skalicky *et al.*, 1980).

The objective of this paper is to indicate how the accuracy of BA estimates of individuals from the normal rat populations depends on their age and on the number of parameters in the test battery. In this paper also the size of a sample from the normal rat population is discussed and comparison of BA of the rats with low and high weights is made.

MATERIALS AND METHODS

Animals

The male and female animals used for estimation of BA are a part of the material described in the previous paper (Dyundikova *et al.*, 1981). Wistar rats (not used for reproduction) were housed in plastic cages, six animals in each. The rats' diet consisted of natural products including oats, curds, white bread, oil, combined granulated feed, vegetables or grass (5; 5; 5; 1; 10; 10 g per animal per day, respectively). Water was supplied *ad libitum*. The rats were decapitated on empty stomach. The animals' age was between 5 and 29 months and fixed within ±5 days.

Biological age indices

The parameters used as BA tests characterize the age-related changes of particular systems and functions (cardiovascular system, connective tissue, metal metabolism), the intensity of the processes recognized as "primary" aging (collagen cross-linking, synthesis of altered proteins, decrease of the "active protoplasm mass") or can be considered as a general sign of the whole organism state (weight).

The test battery for the males included four parameters: relative musculus gastrocnemius weight (RMGW), collagen of the heart (HC), heart weight (HW) and copper content of aorta (ACu). The test battery for the females consisted of ten parameters: body weight (BW), musculus gastrocnemius weight (MGW), RMGW, HW, relative heart weight (RHW), HC, aorta collagen (AC), total skin collagen (TSC), labile skin collagen (LSC), thermolability of liver glucose-6-phosphatedehydrogenase (TE). The reason for the choice of these indices and the methods of their determination were described earlier (Dyundikova *et al.*, 1981).

Estimation of biological age

In accordance with the multiple regression model the rat's BA was calculated by the equation:

$$BA_i = b_o + b_1X_{1(i)} + b_2X_{2(i)} + b_jX_{j(i)} + \ldots b_kX_{k(i)}$$

where BA_i is the estimated (biological) age of the i-th individual; $X_{1(i)}, X_{2(i)} \ldots X_{j(i)} \ldots X_{k(i)}$ are the values of the measured biological parameters 1, 2, . . . ,j, k of the i-th individual, k being the number of parameters (independent variables); b_o, b_1, $b_2 \ldots$, b_j, b_k are the coefficients of the equations calculated on the basis of measurement of all k parameters of each of n individuals in the sample considered. The chronological age y_i (an independent variable) of each of the individuals is known.

The coefficients of the multiple regression were calculated from the equations:

$$\sigma_j = \beta_j \frac{\sigma_y}{\sigma_j};$$

$$b_o = \bar{BA} - \sum_{j=1}^{k} \beta_j \cdot \bar{X}_j$$

where β_j is an auxiliary quantity; σ_y is the standard deviation of the dependent variable; $\overline{X}_j$ and σ_j are the mean and standard deviation of the j-th independent variable; $\overline{BA}$ is the arithmetic mean of the calculated ages.

The auxiliary quantity β_j is calculated as follows:

$$\beta_j = \sum_{j=1}^{k} r_{yj} \cdot r_{jj}^{-1}$$

where r_{yj} are the coefficients of correlation between the dependent and the j-th independent variables; r_{jj} are the coefficients of correlation between the independent variables.

The coefficient of multiple correlation R was determined as

$$R = \sqrt{R^2}; \; R^2 = \sum_{j=1}^{k} \beta_j \cdot r_{yj}$$

The statistical significance of the multiple regression was inferred from the F-criterion. F was evaluated from the expression

$$F = \frac{R^2}{1 - R^2} \cdot \frac{n - k - 1}{k}$$

The multiple regression analysis was performed by a computer.

The accuracy of BA estimates was assessed by comparing the average calculated age of a group of animals at the same age with the chronological age (the Student's *t*-test). The Spierman's coefficient of rank correlation r_s was calculated from the expression

$$r_s = 1 - \frac{6\Sigma d^2}{n(n^2 - 1)}$$

where d is the difference of the ranks of the individual's BA calculated in the two cases; n is the animal number.

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RESULTS

Estimation of the male rat's biological age

The four parameters were determined for each of 23 males (5–29 mos.): RMGW, HC, HW, CuA. The individual values of the measured parameters are shown in Fig. 1 and the simple coefficients of correlation are given in Table 1. Three of the four parameters correlated with age reliably, two of them, RMGW and HC, exhibited quite good (more than 0.6) correlation. The coefficient of multiple correlation between the age and all the four parameters was 0.841. Individual BA (in mo) of the males as a function of the four parameters was expressed by the equations:

$$BA = -21.09 - 22.17 \cdot RMGW + 8.49 \cdot HC + 17.21 \cdot HW - 0.51 \cdot CuA \quad (I)$$

This equation is statistically significant: $F = 10.89$ with d.f. 18 and 4 ($p < 0.01$).

The mean values for four more or less uniform age groups were found with BA of the animals calculated from equation (I). These data are presented in Table 2. Mean BA in the youngest group appeared to be somewhat higher than the chronological age, being much lower in the oldest group; these differences are significant only for the group of old rats. Good agreement of the mean chronological age and mean BA is observed in the 20–23 months group. In the group of 12-month-old rats mean BA is higher than the chronological age but these differences are not certain; if one animal with very high BA

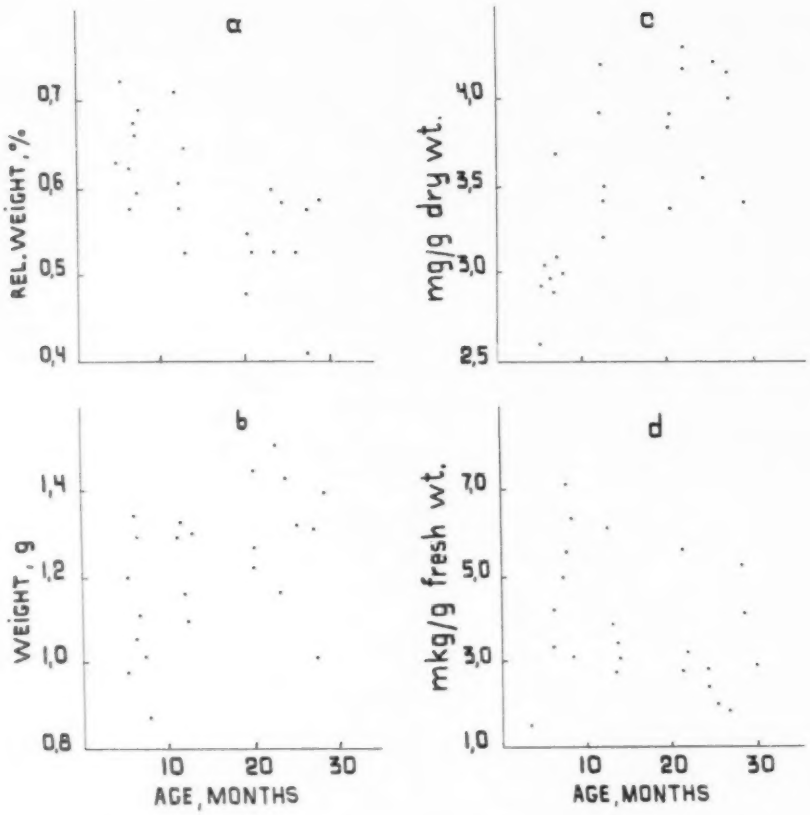

FIG. 1. Individual values for male rats: a—relative m.gastrocnemius weight, b—heart weight, c—heart collagen (hydroxyproline), d—aortic copper.

(21.6 mos.) is rejected, mean BA and mean chronological age will agree better in that group.

Estimation of the female rat's biological age

The following 10 parameters were determined in each of 52 female rats (6–28 mos.): BW, MGW, RMGW, HW, RHW, HC, AC, TSC, LSC, TE. Figures 2 and 3 show the

TABLE 1. CORRELATION MATRIX FOR MALE RATS.

	RMGW	HC	HW	ACu
Age	−0.662*	0.726*	0.484**	−0.329
RMGW		−0.649*	−0.325	0.110
HC			−0.129	−0.307
HW				−0.150

*$p < 0.01$
**$p < 0.05$

TABLE 2. BIOLOGICAL AGE OF MALE RATS.

Age group, months	Animal number	Chronological age, months, mean ± se	Biological age, months mean ± se
5–7	8	6.1 ± 0.28	7.3 ± 1.09
12–12.5	5	12.2 ± 0.12	15.7 ± 1.71
2–23	5	21.3 ± 0.70	21.6 ± 1.54
24–28.5	5	26.4 ± 0.76	20.8* ± 1.62

Significances of the differences by Student's t-test as compared with the chronological age; *$p < 0.02$.

measured parameters of each of the animals. The coefficients of correlation with age and with each other are given in Table 3. Three of the ten parameters (RMGW, RHW, TSC) did not correlate with age, five had low but reliable correlations with the age (BW, MGW, HW, AC, TE). The high age correlation was found for parameters of the connective tissue state (HC and LSC). The multiple correlation coefficient of all 10 parameters with age

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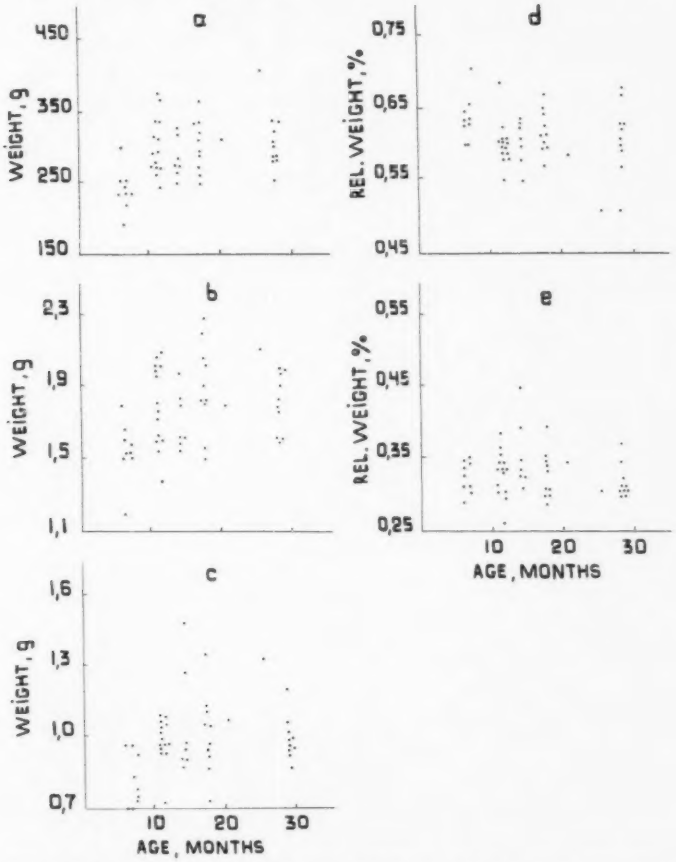

FIG. 2. Individual values for female rats. Weight: a—body, b—m.gastrocnemius, c—heart. Relative weight: d—m.gastrocnemius, e—heart.

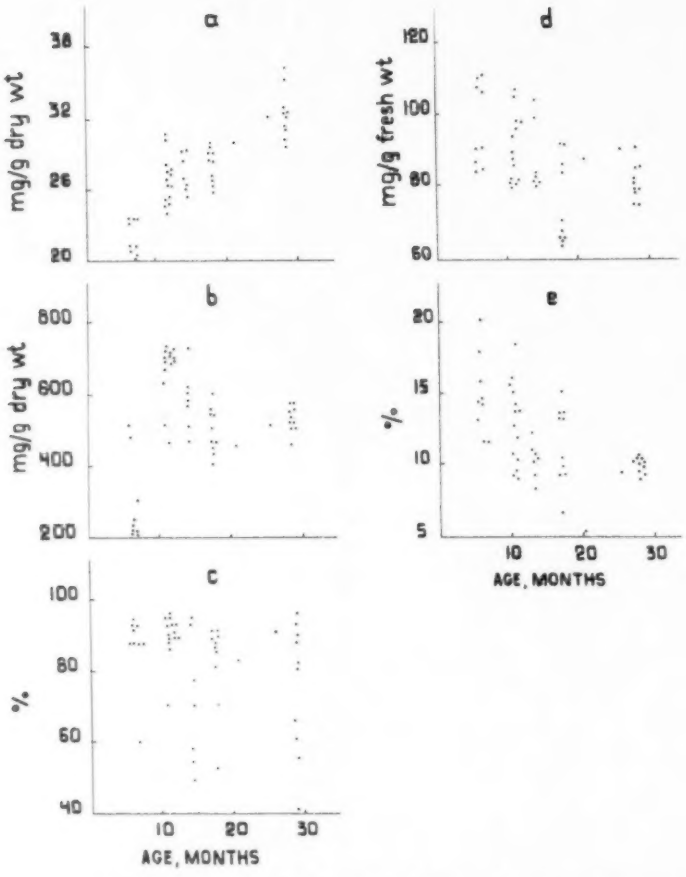

FIG. 3. Individual values for female rats. Collagen: a—heart, b—skin (total), d—aorta, e—skin (labile), c—thermolability of liver glucose-6-phosphatedehydrogenase.

was 0.911. The multiple regression equation which showed a linear relation of age (in mo) to all 10 parameters is of the form:

$$BA = -21.07 + 0.002 \cdot BW - 17.33 \cdot MGW + 73.97 \cdot RMGW + 35.94 \cdot HW - -131.44 \cdot RHW + 1.635 \cdot HC + 0.005 \cdot AC - 0.006 \cdot TSC - 0.313 \cdot LSC - -0.093 \cdot TE \quad (II)$$

According to F-test the equation is reliable with $p < 0.01$ ($F = 20.09$, d.f. 41 and 10).

Rejection of three parameters (RMGW, RHW and TSC) which do not correlate with age and of two parameters (HW and AC) which do not satisfy the requirement of unidirectional age changes (Dyundikova *et al.*, 1981) yields the following expression for age with five parameters:

$$BA = -20.90 - 0.043 \cdot BW + 7.625 \cdot MGW + 1.574 \cdot HC - 0.252 \cdot LSC - -0.090 \cdot TE \quad (III)$$

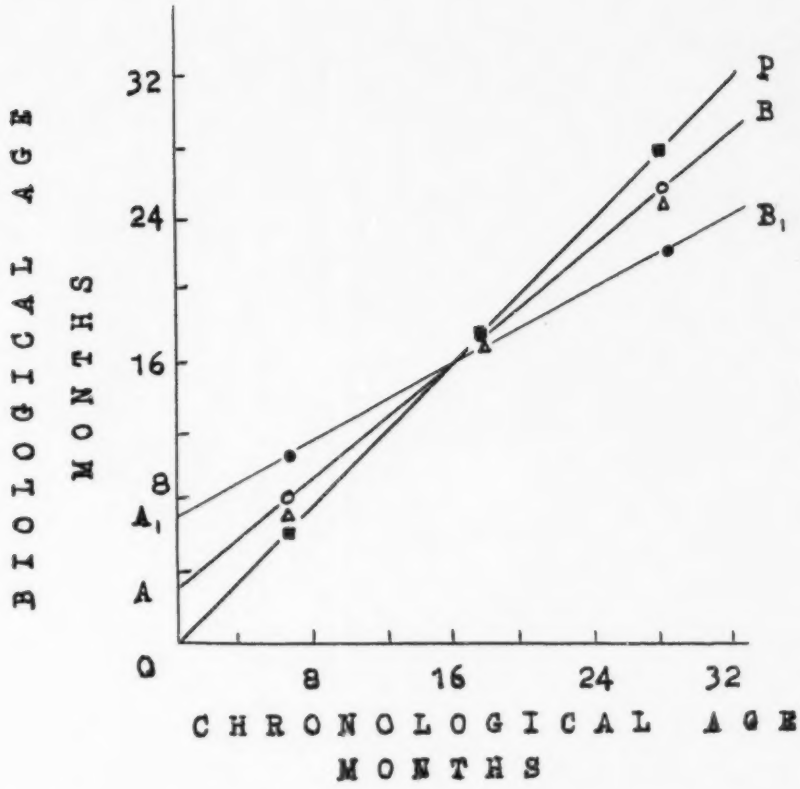

FIG. 4. BA versus chronological age regression lines and mean BA values for 6.7, 17.5 and 28 mo. old female rats: OP (■)—according to the BA concept; AB (○)—predicted for 52 female rats (BA = 2.69 + 0.83y); A_1B_1 (●)—hypothetical with a smaller slope; △—real means.

The coefficient of multiple correlation of the five parameters with age is 0.887; the equation is significant at a high level ($F = 34.01$; d.f. 46 and 5; $p < 0.001$).

Individual BA of each of 52 females was calculated from equations II and III. BA averages estimated for the groups with uniform chronological age are given in Table 4. The table shows that when individual BA in three middle groups (11–12, 14.5 and 17.5 mos.) was calculated with 10 parameters, BA averages and the chronological age agree well. In the young-age group mean BA is somewhat higher than the chronological age, being essentially lower in the old-age group.

When BA was evaluated with five parameters (equation III), BA averages in the three middle groups differ more essentially from the chronological age than in the case of ten parameters. In the old-age group mean BA is considerably lower than the chronological age as is the case with equation (II).

Comparison of individual BA estimates in relation to different sizes of samples

An arbitrary sample of 25 female rats which represents the main population of 52 animals in its age composition, was selected, the randomization rules being observed. The

TABLE 3. CORRELATION MATRIX FOR FEMALE RATS.

	BW	MGW	RMGW	HW	RHW	HC	AC	TSC	LSC	TE
Age	0.371*	0.327**	−0.170	0.277**	−0.079	0.850*	−0.424*	0.167	−0.572*	−0.292**
BW		0.884*	−0.462*	0.799*	−0.075	0.410*	−0.249	0.383*	−0.352*	0.008
MGW			−0.004	0.703*	−0.076	0.292**	−0.335**	0.266	−0.232	−0.055
RMGW				−0.391*	−0.006	−0.345**	−0.054	−0.354*	0.327**	−0.130
HW					0.533*	0.322**	−0.158	0.300**	−0.397*	−0.135
RHW						−0.040	0.100	0.005	−0.151	−0.232
HC							−0.474*	0.365*	−0.548*	−0.089
AC								−0.022	−0.108	0.019
TSC									−0.317**	0.021
LSC										0.255

*$p \leq 0.01$
**$p \leq 0.05$

TABLE 4. BIOLOGICAL AGE OF FEMALE RATS.

Age group, months	Animal number	Chronological age, months, mean ± se	Biological age (months) calculated with	
			10 parameters (equation II), mean ± se	5 parameters (equation III), mean ± se
6–7	9	6.7 ± 0.09	7.9 ± 0.75	6.5 ± 0.74
11–12	14	11.6 ± 0.08	12.2 ± 0.85	13.6** ± 0.84
14.5	7	14.5 ± 0.00	15.6 ± 0.96	16.5† ± 0.94
17.5	10	17.5 ± 0.00	17.2 ± 1.12	17.0 ± 0.98
28	10	28.0 ± 0.00	25.6* ± 0.61	24.8* ± 0.75

Significance of the differences by Student's *t*-test as compared with the chronological age.
*$p < 0.001$
**$p < 0.05$
†$p = 0.056$

multiple regression equation of the age as a function of 10 parameters for a small sample is of the following form:

$$BA = 100.97 - 0.287 \cdot BW - 38.78 \cdot MGW + 149.1 \cdot RMGW + 174.4 \cdot HW - \\ -593.0 \cdot RHW + 1.177 \cdot HC - 0.066 \cdot AC - 0.008 \cdot TSC - 0.471 \cdot LSC - \\ -0.159 \cdot TE \qquad \text{(IV)}$$
$$(R = 0.915;\ F = 7.169,\ \text{d.f. } 14 \text{ and } 10;\ p < 0.01)$$

Individual BA estimates calculated for each of 25 animals from the basic equation II and from equation IV differed in the absolute values by 0.1 to 3.8 months. In most cases (17 rats) these differences were within 1.5 months; in six cases they exceeded 2 months; BA estimates of two animals noticeably differed (by 3.6 and 3.8 mos.). Arrangement of all 25 rats according to BA rank order appeared however quite similar with the two calculation methods. This is also supported by Spierman's rank coefficient, which was found to be of 0.93 for 25 pairs of the estimates (Table 5).

Biological age and body weight

In order to estimate whether the size (weight) of the animals has any effect on their BA,

TABLE 5. DIFFERENCES IN THE RANKS OF INDIVIDUAL BIOLOGICAL AGE CALCULATED FOR 25 FEMALE RATS BY TWO MULTIPLE REGRESSION EQUATIONS WITH DIFFERENT SAMPLE SIZES.

d (rank difference)	Frequency
0	5
1	9
2	2
3	4
4	2
5	1
6	2

$d^2 = 182$; $r_s = 0.93$

"light" and "heavy" animals were selected among the females of 11 to 17.5 months. Because the average weight of all 52 females was 293 g, the standard deviation being ±43 g, the rats with a weight of 260 g and lower were treated as "light," those weighing 320 g and above were referred to as the "heavy" animals.

Since the weights of the body and of the organs enter into the multiple regression equation (II) for female rats and BW, MGW and HW positively correlate with age, it seems natural to expect that BA estimates which include the weight data, would be higher for the heavy rats than for the light animals. A direct effect of weight on BA estimates was eliminated with the aid of the multiple regression equation (for 52 females) which expresses the age in terms of six parameters. Five of them have no direct relation to the body sizes (the connective tissue state parameters and enzyme thermostability); the sixth parameter, RMGW, though being determined from the weight data, is a measure of "active protoplasm mass" (Shock, 1962; Barrows *et al.*, 1962). The following equation was obtained:

$$BA = -25.54 + 16.68 \cdot RMGW + 1.68 \cdot HC - 0.004 \cdot AC - 0.007 \cdot TSC - 0.336 \cdot LSC - 0.083 \cdot TE \quad (V)$$

The parameters of the equation are $R = 0.898$; $F = 31.17$, d.f. 45 and 6; $p < 0.001$.

Mean BA calculated from equations II and V for the light and heavy rats are presented in Table 6. The table shows that with the same chronological age the light rats are somewhat younger biologically as compared with the heavy animals. The differences are however insignificant statistically, presumably because of a small number of the light rats. The rat's BA estimated with all 10 parameters (equation II) and with the parameters which have no direct relation to the weight (equation V) appeared almost the same both for the light and the heavy rats.

DISCUSSION

One of the paradoxes of the concept of BA is the fact that its objective estimation requires complicated studies and bulky calculations, though in everyday life BA of any person can be quite easily evaluated visually. A special study has revealed good agreement of the objective and subjective estimations of man's BA (Borkan and Norris, 1980).

While analyzing how the subjective BA estimation arises, it should be recognized that the procedure includes comparison of some signs of an individual (skin state, hair greying, workability, mobility etc.) with certain general ideas of these signs and of the rate of their

TABLE 6. BIOLOGICAL AGE OF FEMALE RATS AT THE AGE OF 11 TO 17.5 MONTHS DIFFERENT IN THEIR WEIGHTS.

Group Description	n	Weight, g mean ± se	Chronological age, months, mean ± se	Biological age (months) calculated by: equation (II) including weight, mean ± se	Biological age (months) calculated by: equation (V) without weight, mean ± se
Light	5	252 ± 3.7	14.6 ± 1.29	12.4 ± 1.67	12.4 ± 1.61
Heavy	11	343 ± 6.1	14.3 ± 0.84	15.0 ± 0.92	14.7 ± 1.01
All rats of 11-17.5 mo	31	300 ± 7.0	14.2 ± 0.47	14.6 ± 0.68	14.7 ± 0.68

age changes, which we have developed from our experience. The individual BA obtained in such a way expresses a certain general estimate of consistency of different physiological functions of that individual with the statistical age "norms" for the population considered. Hence, the individual's BA is measured by such a chronological age of an average member of the population, with which the physiological parameters of the individual are most consistent (Dubina and Razumovich, 1975).

In the above definition the organism's BA is thought of as a set of the quantities, each reflecting the functional state (the functional age) of some physiological system or a particular function. That the individual's BA is expressed via comparison with the population, to which the individual belongs, is the second specificity of the definition. The mathematic apparatus of multiple regression used at present for BA estimation is consistent with the above two characteristics. Individual BA estimated according to the multiple regression model is, first, the sum with each component dependent on the respective parameter of an individual; second, it expresses a relation of an individual with the population since the coefficients at each of the components of the sum are assessed on the basis of measuring the whole population (or its representative sample).

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Thus, the multiple regression model is theoretically consistent with the main concept of BA. Linearity of this model is also consistent with age-dependent decrease of the main vital functions, although in recent years some authors are inclined to the exponential form of the age-related changes (Comfort, 1979; Skalicky *et al.*, 1980). Applicability of the multiple regression model for comparison of mean BA of differing human subpopulations or for estimation of the efficiency of the influences on aging of experimental animals was demonstrated in a number of studies (Furukawa *et al.*, 1975; Webster and Logie, 1976; Dubina *et al.*, 1979; Skalicky *et al.*, 1980). In order that the multiple regression method would actually be a useful tool in the studies of interference with aging, it is necessary to know its potentialities and restrictions, primarily regarding its accuracy.

Some idea of the accuracy of any method of BA estimation may be gained from comparison of the mean BA of a group of individuals at the same age with their chronological age. The biological age concept assumes that in a normal population the individuals with normal aging rate are of BA close to the chronological age, and the individuals with higher or lower aging rates should be biologically "older" or "younger" than their chronological age, respectively. Mean BA of all individuals at the same age in the normal population should be generally equal to their chronological age. Therefore, coincidence of average BA of the group of individuals at the same age with their chronological age may be used as a measure of accuracy of BA estimates.

As is shown by our data, average BA of the rats in those age groups which are at the middle position or near it, is indeed close to the chronological age. As a rule, in the extreme age groups average BA differs from the chronological age: in the old-age group it is lower, being higher in the young-age group. Such a distortion does not result from inaccuracy due to a small number of animals in the age groups. The same effect was described for a large population in the studies of women's BA using the multiple regression model (Webster and Logie, 1976). The above distortion of BA estimated for extreme groups is a property of linear regression (Fig. 4). It implies that individual BA estimates at the regression edges are assessed with certain errors as compared with the true BA, the errors increasing with the distance of the chronological age of the individual from the regression center.

Thus, accuracy of BA estimates according to the multiple regression model depends on the individual's chronological age, or rather, on the position of the chronological age in the regression. This is not generally an obstacle for comparison between average group BA of experimental and control animals, since with the same chronological age the error is the same. Nevertheless, while calculating BA according to the multiple regression model, it seems more useful to choose the age range of the intact animals so that the treated group would occupy in their chronological age a middlemost position. When exact individual BA is necessary to know, and especially if its changes are to be followed in a longitudinal study, multiple regression analysis fails to give satisfactory results.

Individual's BA reflects adaptive abilities of an organism which in turn depend on the state of many systems and functions and on the manifestation of aging at various organization levels. It is clear that the more diversified physiological age-dependent parameters are included in the test battery, the more accurate is the individual's BA estimate (Comfort, 1979). However, simultaneous determination of many parameters of every animal is difficult in practice (Dubina and Zhuk, 1979; Skalicky *et al.*, 1980). Therefore, the question on the necessary and sufficient number of the parameters arises.

Figure 4 shows that the larger is the coefficient of multiple correlation between the age and the parameters studied, the higher is the accuracy of BA estimates. In the assessment of BA of male Sparague-Dawley rats according to the multiple regression model, Skalicky *et al.* (1980) used 23 parameters and obtained a very high multiple correlation ($R = 0.963$). In the present paper we used the multiple regression equation of age with 10 parameters for Wistar females and obtained the multiple correlation $R = 0.911$. Rejection of the parameters which bear little information, of which three (RMGW, RHW, TSC) do not correlate with age and two (HW, AC) are not consistent with the criterion of unidirectional age-related changes, reduced R to 0.887, the accuracy of BA estimates being somewhat lower. Finally, in estimation of Wistar males' BA we used only four parameters ($R = 0.841$).

It seems possible to calculate or take by convention certain R as a minimal value and proceed then to the problem of the number of parameters sufficient for BA estimation with certain accuracy. This seems however not only a problem of mathematics but primarily a conceptual one. Evaluation of the aging status of organism is likely to include at least as many parameters as there exist independent mechanisms of aging (or/and more or less independent ways of their realization). Before this central problem of gerontology is solved, the choice of any number of parameters for BA estimation would depend on the worker.

So that estimation of BA could be used in the studies on the life prolongation of experimental animals as a measure of interference efficiency, it is necessary that the method employed would not exceed the cost of the survival studies. This in turn gives rise to the problem of the sample size from the intact animal population to establish the "normal aging standard."

As is shown by the present data, the statistically significant multiple regression equations were obtained for 23 male and 52 female rats. Besides, 25 animals were chosen from all the females at random. The selection was performed separately in each age group of the animals so that a small sample corresponded to the main one in the age composition. The multiple regression equation of age with 10 parameters for 25 females was also statistically significant. Comparison of the individual BA obtained for each of the 25 rats

by the basic expression and by the small sample formula shows that the two estimates are very similar. This suggests that in spite of random variation of the parameters, the main trends of their age-related changes are displayed also in as small a sample as 25 animals.

Thus, if quite reliable parameters are taken as BA tests, the size of a sample from an intact animal population to establish the "normal aging standard" may be small (25–30 animals). As a measure of the reliability of BA tests, relatively small variations of the age correlation coefficients in samples of different sizes could be taken. As an example, we may compare the age correlations of four parameters in a large sample (30–68 male rats, Dyundikova *et al.*, 1981) and in a small sample (23 males, this paper). The following r were obtained for the small and the large samples, respectively: RMGW = −0.662 and −0.614, HW = 0.484 and 0.379, HC = 0.726 and 0.569; CuA = −0.329 and −0.376.

It is known that excess weight of man is a very substantial prognosing parameter which determines a high death probability (Taton, 1981). The same was demonstrated for rats: long-time dietary restriction results in a lower weight and noticeably increases the lifespan (McCay *et al.*, 1956; Ross, 1978). With free choice of the amount and composition of the food the rats who consumed it in smaller amounts ("slowly growing") have a longer lifespan (Ross *et al.*, 1976). These facts suggest that the rats with a low weight should have lower BA than their chronological age, and reversely, BA of heavy rats must exceed their chronological age. This suggestion was checked by comparison of middle-age female rats in extreme weight categories. Contrary to the expectation, there were no differences between mean BA of the heaviest rats and their chronological age. BA of the lightest rats was indeed somewhat lower but not appreciably.

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It is quite possible that the relationship between the weight and BA of rats cannot be revealed with as small a sample as ours. Besides, some of the animals with a low weight can, in fact, have, very probably, a long lifespan, whereas the other light animals suffer from latent diseases and are to die in the nearest future. Their BA may be high, masking the relationship discussed. These differences can be distinguished by longitudinal observations of the animal weights. According to Economos and Miquel (1980) the weight variations in a population are so closely related to vitality that it can be used for survivability forecasting. The weight-BA relations seem to deserve more detailed investigation.

SUMMARY

The physiological parameters of 23 male and 52 female Wistar rats at the age of 5 to 29 months have been used to assess biological age (BA) based on multiple regression analysis. Test batteries included the following indices of BA: relative musculus gastrocnemius weight, heart weight, heart collagen, aorta copper content for males; body weight, absolute and relative musculus gastrocnemius and heart weights, heart collagen, aorta collagen, total and labile skin collagen, thermolability of liver glucose-6-phosphatedehydrogenase for females. It is shown that judging from the coincidence of mean BA of a group of individuals at the same age with their chronological age, the assessment accuracy is higher in the regression center, while BA estimates appeared overestimated and underestimated for younger and older animals, respectively. BA estimates for the females obtained with 10 parameters have shown better accuracy as compared with 5 ones, though the rejected parameters were not consistent with the validity criteria for BA tests. It is sug-

gested that the question of the necessary and sufficient number of physiological parameters for BA estimation may be answered provided some fundamental problems of the aging mechanisms would be solved.

A random sample of 25 animals with the age composition corresponding to the main sample was selected from the females. The multiple regression equation with 10 parameters for a small sample appeared statistically significant and BA estimates for 25 rats calculated from that equation and from the equation for the main sample (n = 52) were similar. This fact as well as a possibility to assess BA of 23 males have led to a conclusion that aging of a normal rat population can be characterized with the aid of the multiple regression equations using a small sample (25–30 animals).

Comparison of BA averages of female rats in extreme weight categories has not revealed reliable differences for the lightest and heaviest animals.

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EFFECTS OF ALCOHOL ON THERMOREGULATION IN AGED MONKEYS

M.T. MURPHY and J.M. LIPTON

Physiology and Anesthesiology Departments, Southwestern Medical School,
Univerity of Texas Health Science Center at Dallas, Dallas, Texas 75235

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Abstract—Ethanol ingestion has been implicated in accidental hypothermia of the elderly, but there is no prior data on the relative sensitivity to alcohol of aged homeotherms that might account for disproportionate dysthermia in this age group. To assess their sensitivity, ethanol (0.5-2.0 g/kg) or H_2O was given by gavage to squirrel monkeys less than 4 yrs old, 4–9 yrs of age and over 9 yrs old. Dose-related decreases in body temperature occurred in all three groups in a 25.5°C environment, with the greatest decreases in the oldest animals. In an 18.5°C environment the hypothermias caused by 0.5 and 1.0 g/kg ethanol were greatly augmented in old monkeys and in some cases their temperatures fell to potentially life-threatening levels. In a 30.5°C environment, 1.0 g/kg ethanol given to monkeys caused approximately equal temperature reductions in the three groups. When determined after 1.0 g/kg ethanol, peripheral vasomotor tone increased significantly in animals of each age group in all three environments. Decreases in rectal temperature were associated with parallel decreases in oxygen consumption. These results indicate that: 1) aging is associated with an increased sensitivity to the hypothermic action of ethanol in thermoneutral and cold environments, 2) ethanol in the doses tested inhibits heat production mechanisms without suppressing compensatory vasoconstriction in response to decreased body temperature.

Key Words: aging, alcohol, hypothermia, heat production, squirrel monkey

INTRODUCTION

THE AGED account for a large portion of the victims of accidental hypothermia (Butler and Shalowitz, 1978; Vaisrus, 1978; MacMillan, Corbett, Johnson, Smith, Spalding and Warner, 1967; McNicol and Smith, 1964). Drugs are often implicated in this type of dysthermia in the elderly (Butler and Shalowitz, 1978; Vaisrus, 1978), and recent basic research on chlorpromazine, diazepam and taurine (Lipton, Whisenant and Gean, 1979; Clark and Lipton, 1981a,b) suggests that the sensitivity to these temperature-altering compounds markedly increases with age. Clinical reports (Lipton, 1983; Hirvonen, 1976; Weyman, Greenbaum and Grace, 1974) indicate that alcohol consumption, a primary abuse problem in the aged, is a major factor in accidental hypothermia. Despite the relationships between aging, alcohol and body temperature, it is not known if alcohol contributes more to accidental hypothermia in aged homeotherms by virtue of an increased

Please address correspondence to: J.M. Lipton, Physiology Dept., Southwestern Medical School, UTHSCD, Dallas, TX 75235.

susceptibility to the drug with age. This idea was explored in the present experiments by administering ethanol to young, adult and aged subhuman primates while they were exposed to thermoneutral, cold and hot environments.

There is no unanimous agreement concerning the nature of alcohol-induced changes in body temperature. To explain decreases in body temperature caused by alcohol, research on laboratory animals and on human volunteers has implicated: 1) general CNS depression (Hirvonen, 1979), 2) a more specific, limited disruption of temperature control (Myers, 1981), 3) a change in thermoregulatory set-point (Lomax, Bajorek, Chesarek and Chaffee, 1980), 4) a direct peripheral vasodilitation or inhibition of vasoconstriction (Ritchie, 1980), 5) impairment of shivering thermogenesis (Graham and Baulk, 1980) and 6) hypoglycemia (Haight and Keatinge, 1973). Because of these divergent opinions heat production and peripheral vasomotor tone were measured, in some of the present experiments, along with core temperature to determine how thermoeffector activity is altered during alcohol-induced hypothermia.

METHODS AND PROCEDURE

Animals

Male and female squirrel monkeys (Saimiri sciureus) 3–16+ yrs old and 500–1100 g in weight were used. Birthdates or, in the case of some animals over 12 yrs old, the dates of importation were documented. Imported animals were known to be over 1 yr old at the time of importation. All animals were free of tuberculosis, and only those in good health were used in the experiments. Animals were individually caged in a 23–25°C environment with a 12-hr light-dark cycle and had lived in the environment for at least 3 months prior to testing. They were maintained on monkey biscuits (Wayne, Allied Mills, Inc.) and fruit; water was available *ad libitum*. Before testing was begun, a thermistor probe (Yellow Springs International No. 701, 10 cm insertion) was inserted into the rectum and each monkey was trained to sit for 8-hr periods in a restraining chair designed for the species (Plaslabs, Inc.). Experiments were performed during the light cycle and started in the morning before daily feeding.

The monkeys were divided into three age categories: young (< 4 yrs), adult (4–9 yrs) and aged (> 9 yrs). The division between adult and aged animals at nine yr was to assure that the responses of definitely aged monkeys were used in the comparisons (Clark, Gean and Lipton, 1980).

For each test, a monkey was placed in a restraining chair located within an environmental chamber maintained at 18.5, 25.5, or 30.5 ± 0.5°C. In certain experiments skin temperature was recorded using a thermistor probe (Yellow Springs International No. 709B) taped to the shaved dorsal surface of the base of the tail. Both rectal and skin temperatures were recorded at 10 min intervals using a digital temperature recorder (Datalogger, United Systems Corp.) connected to a MINC 11 online computer (Digital Equipment Corp.). In some experiments oxygen consumption was monitored with an oxygen analyzer (Beckman Instruments, model OM 11) and recorded on a polygraph machine (Grass Instruments), using procedures described previously (Lipton and Kennedy, 1978).

Solutions and administration

Alcohol was administered after recording base-line temperature for 1.5 hr or more, and temperatures were monitored for 5 hr after ethanol administration. Absolute alcohol (ethanol) was diluted with distilled water to a total volume of 5 ml. Doses for each animal were 0.5, 1.0, and 2.0 g ethanol/kg body weight, and 5 ml distilled water was given as a control. The solutions were delivered intragastrically to the unanesthetized animals using a number 8 French infant feeding tube inserted 15 cm past the incisors. The alcohol and control injections were administered in two doses of 2.5 ml 20 min apart to reduce vomiting. Doses were administered in random order and were warmed to approximately 36°C prior to administration. All experiments were separated by at least 72 hours.

Calculations

The thermal response index (TRI), the area under the temperature curve expressed as Δ°C $\times$ hr (Clark and Cumby, 1975) was used to quantify the total effect of ethanol on body temperature. Values are expressed as mean change ($\pm$SEM) in TRI after subtraction of control TRI scores. For most graphs cumulative TRI scores

were calculated for each hr of the 5-hr recording period. The least significant difference (LSD) one-tailed test (Kirk, 1968) was used for statistical analysis of the data.

RESULTS

Effects of alcohol on body temperature

Ethanol produced dose-related decreases in core temperature in all three age groups. Temperatures began to fall 30–60 min after ethanol administration, a delay corresponding to that found previously in humans for absorption of the drug when given orally (Kater, 1969). The lowest temperatures were recorded 1.5–2.0 hr after administration, a time interval previously associated with maximum blood alcohol concentration after oral administration in primates (Pieper, 1973). In all three ambient temperatures animals over nine yrs of age developed the greatest hypothermia after each dose (Figs. 1–3, Table 1). In the 25.5°C environment young (< 4 yrs) and adult (4–9 yrs) monkeys developed only slight, if any, decreases in core temperature after 0.5 g/kg ethanol. After the 1.0 and 2.0 g/kg doses adult animals developed greater hypothermias that differed only slightly from those produced in the aged animals ($\alpha = 0.15$).

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The greatest decreases in core temperature in these experiments occurred in the aged monkeys after 1.0 g/kg ethanol in the cold environment (Fig. 2, Table 1). Base-line temperature in the cold was lower in these animals, and some had to be removed from the chamber and warmed when rectal temperature fell below 35°C after the 0.5 and 1.0 g/kg doses. For this reason 2.0 g/kg ethanol was not given in the cold. In this environment increasing the dose from 0.5 to 1.0 g/kg augmented hypothermia in animals 4–9 yr old. In the young animals 1 g/kg ethanol caused only a slight hypothermia, in contrast to the small increase in temperature after the 0.5 g/kg dose. Only the intermediate dose of ethanol (1.0 g/kg) was tested in the hot environment. This dose induced approximately the same decrease in core temperature in all three age groups (Fig. 3, Table 1).

Aged primates not only developed greater decreases in core temperature than did younger animals, but the hypothermic effect of each ethanol dose was more prolonged. Recovery from hypothermia began in the young animals about 3 hr after ethanol ad-

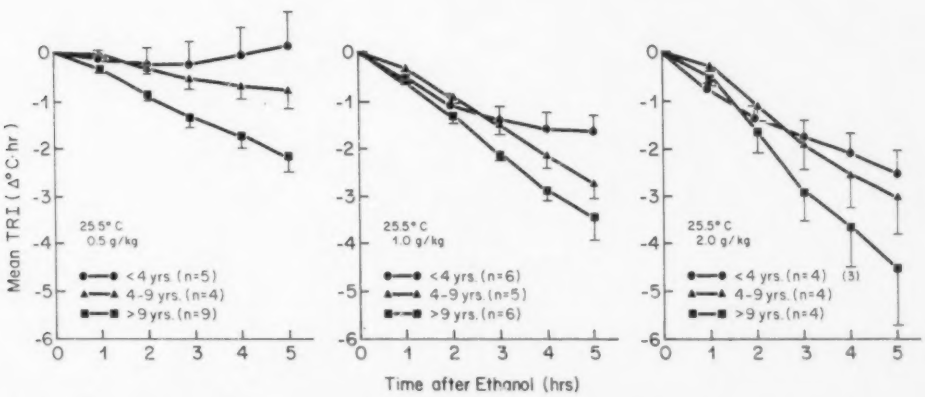

FIG. 1. Change in rectal temperature of young, adult and aged monkeys after ethanol (0.5–2.0 g/kg) in a thermoneutral environment. Values are mean changes (±SEM) in cumulative TRI from control.

TABLE 1. CHANGE IN RECTAL TEMPERATURE (°C) OF YOUNG, ADULT AND AGED SQUIRREL MONKEYS PRODUCED BY INTRAGASTRIC ETHANOL IN THERMONEUTRAL, COLD AND HOT ENVIRONMENTS. (VALUES FOR EACH AGE GROUP ARE TOTAL MEAN CHANGE IN TRI (±SEM) FROM CONTROL OVER THE 5 HR RECORDING PERIOD.)

Ambient Temperature	18.5°C		25.5°C			30.5°C
Dose	0.5 g/kg	1.0 g/kg	0.5 g/kg	1.0 g/kg	2.0 g/kg	1.0 g/kg
Age Group						
< 4 yrs	+1.50 ± .84	−1.06 ± .22	+0.16 ± .72	−1.64 ± .34	−2.52 ± .48	−1.64 ± .44
4–9 yrs	−0.38 ± .12†	−2.77 ± .70†	−0.80 ± .38	−2.74 ± .35†	−3.02 ± .79	−1.66 ± .29
> 9 yrs	−2.51 ± .32†*	−4.42 ± .88†*	−2.16 ± .33†*	−3.43 ± .50†	−4.53 ± 1.20†*	−1.60 ± .24

†Significantly different from > 4 yrs ($\alpha = 0.05$ or better)
*Significantly different from 4–9 yrs ($\alpha = 0.05$ or better)

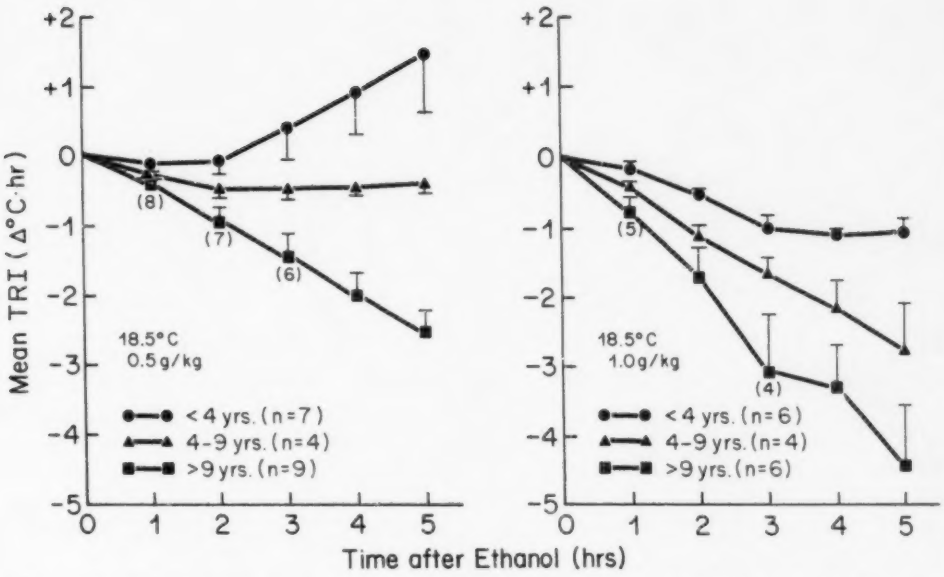

FIG. 2. Change in rectal temperature of young, adult and aged monkeys after ethanol in a cold environment.

ministration. Adult monkeys showed intermediate responses, with some recovery of core temperature after the 0.5 g/kg dose, but none after 1.0 g/kg ethanol. Core temperature of the aged animals remained low throughout the 5-hr recording period after each dose.

Effects of ethanol on heat loss and heat production

Hypothermia produced in animals of all three age groups by 1.0 g/kg ethanol was ac-

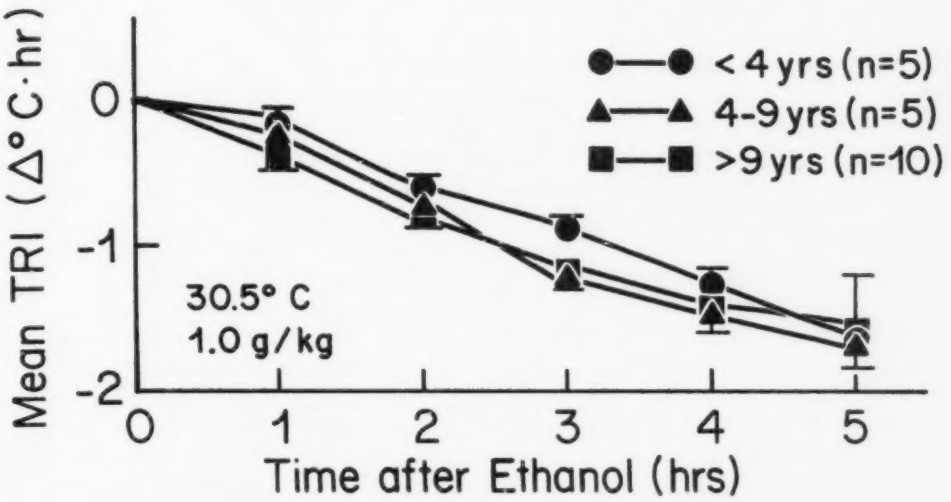

FIG. 3. Change in rectal temperature of young, adult and aged monkeys after ethanol in a hot environment.

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TABLE 2. TOTAL CHANGE IN TAIL SKIN TEMPERATURE AND O_2 CONSUMPTION OF REPRESENTATIVE MONKEYS AFTER ETHANOL IN THERMONEUTRAL, HOT AND COLD ENVIRONMENTS.

Ambient Temperature	*Tail Skin Temperature* Mean TRI (Δ°C • hr)	*O_2 Consumption* (Δ ml O_2 • kg^{-1} • min^{-1})
25.5°C	−8.56 ± .91	−5.00 ± 2.85
30.5°	−3.15 ± .38	−7.98 ± 2.07
18.5°	−3.84 ± .31	−10.57 ± 2.22
	n = 10	n = 4

companied by vasoconstriction. Table 2 shows change in tail skin temperature, a reflection of peripheral vasomotor tone, after ethanol administration in representative animals drawn from the three age groups and exposed to the three ambient temperatures. Vasoconstriction paralleled the fall in core temperature in all three environments. No marked difference in the vasoconstriction responses among young, adult and old animals occurred at any of the three environmental temperatures. No shivering was observed in ethanol-treated monkeys exposed to cold.

To assess the effects of ethanol on heat production and vasomotor tone simultaneously animals from each age group were tested at the three ambient temperatures. In all animals and in all three environments 1.0 g/kg ethanol decreased oxygen consumption, as well as tail and deep body temperature (Fig. 4, Table 2).

DISCUSSION

In the present study oral administration of ethanol caused dose-related decreases in core temperature of subhuman primates. Adult and aged monkeys developed significantly greater hypothermia than did younger animals after each dose tested, suggesting that changes occur in susceptibility to the temperature-altering effects of this drug with aging. Similar increases in sensitivity of aged animals to other drugs that alter temperature regulation, such as chlorpromazine, diazepam and morphine (Lipton *et al.*, 1979; Clark and Lipton 1981a; Clark *et al.*, 1982), have been reported. If our results can be generalized to the case of aged man, alcohol and other psychoactive compounds may contribute to dysthermia in the elderly by virtue of these changes in sensitivity.

In the thermoneutral and cold environments, young and adult monkeys did not respond to 0.5 g/kg ethanol and maintained stable body temperatures, but core temperature of the aged animals dropped considerably. After a dose of 1.0 g/kg, core temperature of adult as well as aged monkeys decreased markedly in all three ambient temperatures. In the 25° and 18°C environments, hypothermia in some aged animals after 0.5 and 1.0 g/kg ethanol reached dangerous levels and necessitated active rewarming to prevent irreversible heat loss and death. Since alcohol abuse is a primary concern in the aged human population, a similarly enhanced hypothermic response to alcohol ingestion in man might contribute significantly to the increased probability of accidental hypothermia in elderly patients, particularly in those exposed to cold. The increased sensitivity to the temperature-altering effects of alcohol and the greater duration of the hypothermic effect of this drug in aged homeotherms suggest that elderly patients admitted to hospitals with any indication of

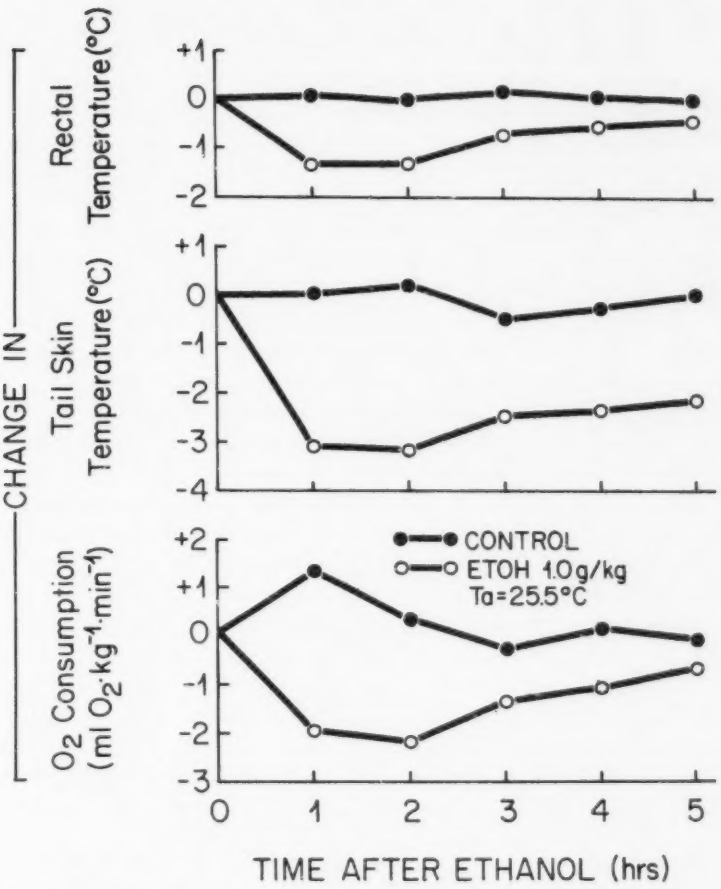

FIG. 4. Change in rectal and tail skin temperature and O_2 consumption of a representative adult monkey after ethanol in a thermoneutral environment.

recent alcohol ingestion should be carefully evaluated for the possibility of accidental hypothermia.

The hypothermic effect of ethanol has been described in previous studies in both the rat (Lomax *et al.*, 1980) and man (Graham and Dalton, 1980; Graham and Baulk, 1980). Although there is considerable clinical and experimental data on alcohol/temperature relations, the mechanism by which alcohol alters thermoregulation has not been fully established. We found that 0.5–2.0 g/kg ethanol, doses comparable to those which produce moderate to high intoxication in man (Ritchie, 1980), significantly reduced core temperature of subhuman primates in thermoneutral, as well as cold and hot environments. The oxygen consumption rate, a measure of overall heat production, also decreased significantly following ethanol administration in each ambient temperature. This might result from a decrease in the thermal set-point (Lomax *et al.*, 1980). However, a lowering of the set-point should initiate coordinated changes in heat-production and heat-loss effectors. Vasomotor tone of the tail, a primary mechanism of temperature control in

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these primates (Stitt and Hardy, 1971), was monitored after 1.0 g/kg ethanol. Distinct vasoconstriction accompanied core temperature decreases at each ambient temperature. It appears that in the squirrel monkey model the pronounced decrease in tail skin temperature reflects a heat-conservation response to the drop in core temperature. This evidence of opposing or compensatory effector activation provides strong evidence that ethanol in the doses tested does not alter the thermal set-point, but rather lowers core temperature primarily through the selective suppression of heat production mechanisms.

Vasoconstriction found in the present experiments does not conform to the generally accepted view of alcohol-induced vasodilitation (Ritchie, 1980; Wallgren and Barry, 1970). Previous studies (Gillespie, 1967; Fewings, Hanna, Walsh and Whelan, 1966) have indeed shown slight vasodilitation after alcohol, but the affected sites seem to primarily be the extremities. Gillespie (1967) found that vasodilitation produced by moderate amounts of alcohol is solely cutaneous. Muscle blood flow decreased or remained unchanged after these doses of alcohol. It is possible that specific cutaneous body sites respond to alcohol through vasodilitation or flushing, while blood flow to other peripheral regions and to the trunk muscle mass remains unchanged or decreases in response to depressed metabolism.

It may be that the doses used in the present experiment exert an intermediate depressive effect on heat production pathways, but leave heat conservation via vasoconstriction intact. Vasoconstriction after these doses may indicate an attempt to compensate for the drop in core temperature resulting from metabolic suppression. After larger doses of ethanol, however, a more generalized depression is likely to occur, ultimately affecting both heat production and conservation mechanisms. Such depression caused by larger doses could quite possibly result in a more widespread reduction of vasomotor tone and the induction of a state of poikilothermia (Myers, 1981). This level of suppression is perhaps present in test animals and humans in which the amount of alcohol ingested induces more severe intoxication than that induced by moderate "social" drinking.

Uncertainty still exists regarding the extent of the vasomotor response and the amount of alcohol necessary to elicit it. Nevertheless, it should be noted that, although present to some extent after alcohol ingestion, vasodilitation should not necessarily be labelled as the main source of heat loss in alcohol-induced hypothermia. Depressed metabolism resulting from the suppression of heat production mechanisms may be the primary cause of decreased body temperature.

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AGING CHANGES IN LYMPHOPOIETIC AND MYELOPOIETIC ORGANS OF THE ANNUAL CYPRINODONT FISH, *NOTHOBRANCHIUS GUENTHERI*

E.L. Cooper,[1] A. Zapata,[2] M. Garcia Barrutia[2] and J.A. Ramirez[3]

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INTRODUCTION

Biologic aging has been analyzed from many different viewpoints (see review by Timiras, 1978). Some investigators (Burnet, 1959; Comfort, 1961, 1963; Walford, 1962) have discussed the possibility that aging may be an immunologic phenomenon, and recently Kay (1978, 1979) has stressed the importance of the thymus as a trigger for human aging. When considering comparative aging in primitive animals, some teleost fish have a relatively short lifespan (about one year), and they thus represent a choice model system for aging studies. Relevant studies using these fish have focused on at least three main parameters. First, there has been an emphasis on laboratory lifespans coupled with an analysis of certain age-associated pathologies (Comfort, 1960, 1963, 1964; Egami and Etoh, 1969; Liu and Walford, 1969; Markofsky and Milstoc, 1979a,b; Walford and Liu, 1965). Second, from a physiological viewpoint, the effects of parameters such as nutrition or hypothermia on fish lifespans have been investigated (Liu and Walford, 1966, 1975). Third, from a histologic viewpoint, morphological changes of various organs and their relationships with aging and sexual maturity have been described (Honma and Tamura, 1972; Tamura and Honma, 1969, 1973, 1974, 1977; Tamura, 1978).

Despite these necessary studies, there is still no systematic review of the relationships between observed changes in fish lymphohemopoietic organs and aging as one basis for the immunologic theory of aging. To begin an analysis of the relationship between aging and the immune system, we have analyzed the morphologic changes in lympho-myeloid organs of the East African annual fish, *Nothobranchius guentheri* and *Cynolebias adloffi*. We also emphasize the possible relationship between changes in the immune system and

[1]Correspondence should be addressed to E.L. Cooper at the Department of Anatomy, School of Medicine, University of California, Los Angeles, California 90024.

[2]Present address: Departamento de Citologia e Histologia, Facultad de Biologia, Universidad de Leon, Leon, Spain.

[3]Present address: Departmento de Immunologia, Escuela, Nacional de Ciencias Biologicas, Carpio y Plan de Ayala, Mexico 17 D.F., Mexico.

appearance of tumors in aged fish. Although not proven, such an observation poses interesting relationships between aging, decline of the immune system, and the development of neoplasia and suggests a breakdown of immune surveillance.

MATERIALS AND METHODS

Fish used for study were *Nothobranchius guentheri* (class Pisces, family *Cyprinodontidae*) a small killifish from East Africa (Total 50). Some specimens of the South American annual fish, *Cynolebias adloffi*, were also examined. The fish were purchased commercially (Mr. R. Van Veen, Los Angeles, CA.), and maintained in our laboratory at 22°C with a 12 hour light/dark photoperiod and a diet of live adult *Artemia salina* supplemented with frozen homogenized beef heart. The fish (5 per each period) were killed at 2 weeks, 1, 2, 4, 6, 8, 9, 10, 11 and 12 months, by immersion *in toto* in Bouin's fluid. Five fish of each stage were processed for routine light microscopy. Transverse sections of the entire fish body were prepared and stained with Masson's hemalum and picroindigo carime.

RESULTS

Normal young immune system

Before describing the histopathologic changes associated with aging of the immune system and the simultaneous appearance of numerous tumor cells in 12 month old fish, it is well to describe briefly the immune system as it appears normally. The *peripheral blood* contains all the leukocytic types which circulate freely. The main lympho-myeloid organs are the *thymus*, *spleen* and *kidney* (*pro- and mesonephros*). The thymus is situated in association with the branchial epithelium, usually at the junction of the operculum and the dorsal body musculature. It is surrounded by connective tissue, and by two months of age, shows a considerable increase in size (Fig. 1). The thymus is packed with thymocytes, but there was hardly any distinction between the cortex and medulla. The spleen is composed of lymphoid and erythroid elements, although the delineation into distinct white and red pulp is not sharp. As early as two weeks, the kidney shows accumulations of hemopoietic cells, increased activity that is quite pronounced at six months (Fig. 2). Since bone marrow is absent in fish, the kidney plays a major role in hemopoiesis.

The senescent immune system: changes in the thymus associated with increased tumor incidence

The earliest changes in the senescent immune system began to appear in the thymus at four months (Table 1). As in most other vertebrates, this was signaled by an increase in the amount of connective tissue elements. Increased connective tissue seemed first to affect the capsule and its extensions inward into the predominantly lymphoid parenchyma. Myoid cells also characterized the thymus at four months, although the presence of these peculiar muscle cells in primitive vertebrates (fish, amphibians, reptiles) is not necessarily associated with aging. By six months, the thymus showed an increase in the number of epithelial cysts and a decrease in lymphocytes.

At nine months, the thymus of *Cynolebias adloffi* showed essentially the same changes as in *Nothobranchius guentheri* up to six months, but this does not imply that the two fish age at the same rate. This probably reflects only the relative degrees of histopathologic changes that occur from normal to total senescence up to the 12-month period. By 12 months, several fish, especially *Nothobranchius*, had thymuses that were completely degenerated when compared with the condition in two-month-old fish (Fig. 3). There were less lymphocytes whose appearance was masked by massive amounts of connective tissue.

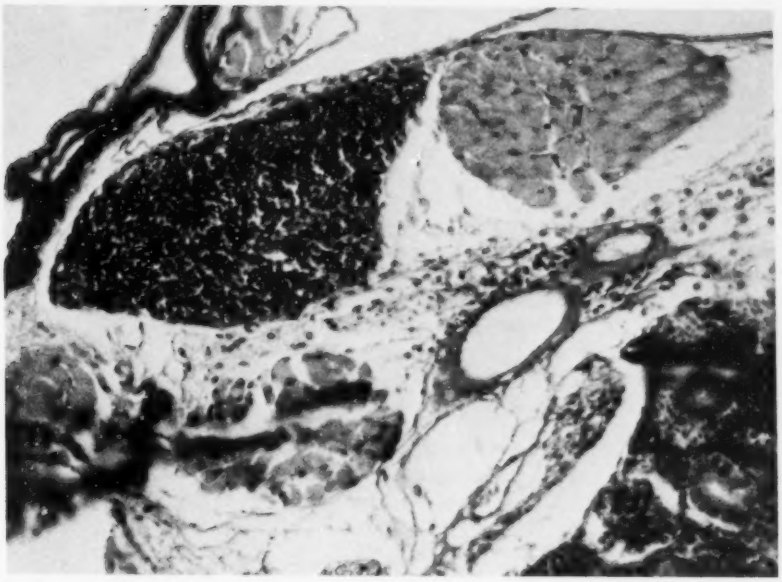

FIG. 1. The thymus showing dense accumulations of thymocytes is located in association with the branchial epithelium usually at the junction of the operculum and the dorsal body musculature. (× 16)

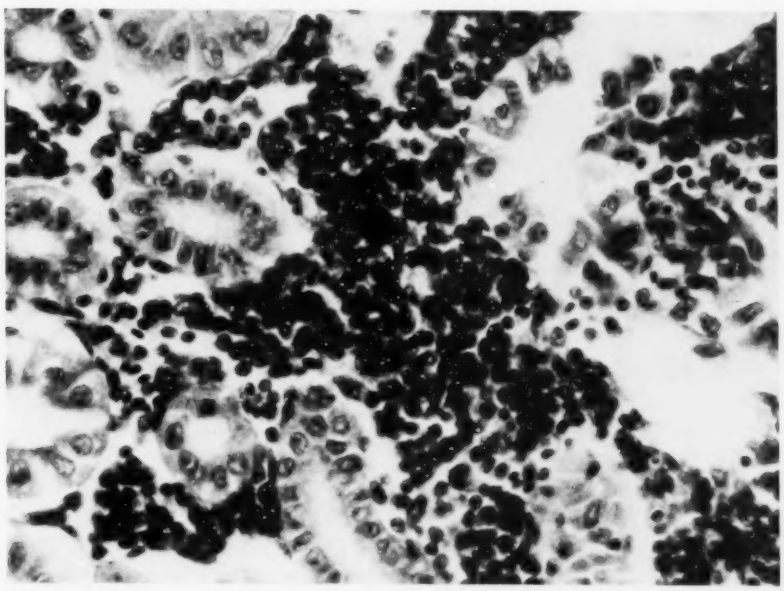

FIG. 2. The kidney showing well-defined tubules with increased hemopoietic activity apparent as numerous cells with darkened nuclei. (× 40)

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TABLE 1. TUMOR INCIDENCE IN RELATION TO AGE AND SENESCENT CHANGES OF THE THYMUS IN *Cynolebias adloffi* AND *Nothobranchius guentheri*

Age of fish (in months) when analyzed for histopathologic alterations; total fish-50; 5 per observation period	Condition of spleen, kidney, liver	Condition of thymus	Tumor incidence (+ = 100%; −0%)
0.5	kidney-hemopoietic; spleen-hemopoietic	highly lymphopoietic	−
1	kidney-hemopoietic; spleen hemopoietic	highly lymphopoietic	−
2	kidney-hemopoietic; spleen-hemopoietic	highly lymphopoietic	−
4	kidney-hemopoietic; spleen-hemopoietic	earliest senescent changes; increased connective tissue; myoid cells	−
6	kidney-hemopoietic; spleen-hemopoietic	increase in epithelial cysts	−
8	kidney-hemopoietic; spleen-hemopoietic	increase in epithelial cysts	−
9	kidney-hemopoietic; spleen-hemopoietic	increase in epithelial cysts	−
10	kidney-hemopoietic; spleen-hemopoietic	increase in epithelial cysts	−
11	kidney-hemopoietic; spleen-hemopoietic	increase in epithelial cysts	−
12	Marked tumorous growth in kidney and liver. Tentative classification in kidney: *histiocytic lymphoma*	Total degeneration essentially devoid of lymphocytes	+ Marked tumorous growth in oral cavity, connective tissue

During the 12-month observation period characterized by beginning and total subsequent degeneration of the thymus, the rest of the tissues and organs were essentially normal in appearance. These tissues and organs included connective tissue, heart, liver, small intestine, testes and gas bladder, and up to six months there was no evidence of tumors.

In contrast, at the time when the thymus showed its greatest senescence at 12 months, there were marked tumorous growths apparent in the oral cavity, connective tissue, kidney and liver. The kidney and liver showed the most pronounced changes. In its normal state, the kidney is composed of rather closely aggregated tubules between which are interspersed hemopoietic cells, often consisting of many lymphocytes. At 12 months, the tubules appeared to be few and were abnormally displaced by massive accumulations of tumor cells (Fig. 4). These tumor cell masses were circumscribed, well-delineated, and usually consisted of a core of large, faintly staining cells. The nuclei were also large and showed pronounced nucleoli and thin threads of chromatin. Pyknotic nuclei were also numerous (Fig. 5). The liver showed a rather similar histopathology. Although characterized by evenly distributed cords of hepatocytes in the normal condition, the tumorous intrusions appeared as circumscribed masses which nearly obliterated the few remaining parenchymal components (Figs. 6, 7).

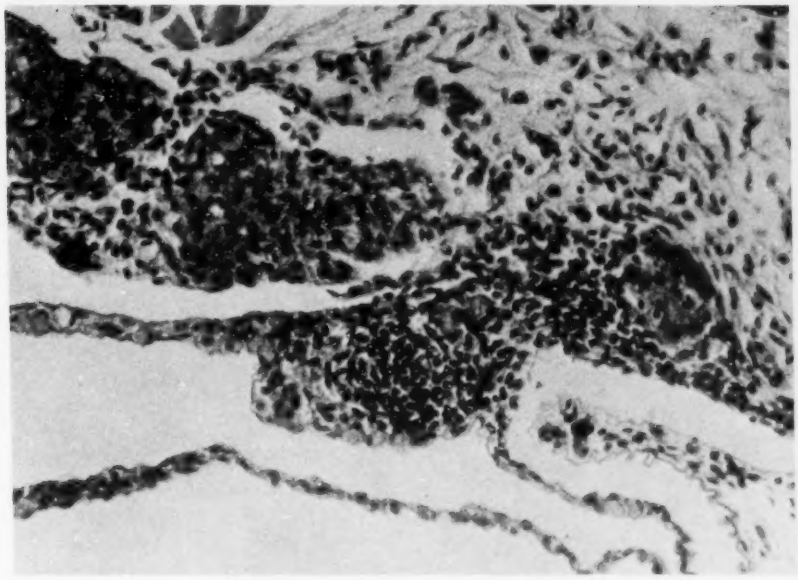

FIG. 3. The thymus at 12 months of *Nothobranchius* showing complete degeneration characterized by few thymocytes. (× 16)

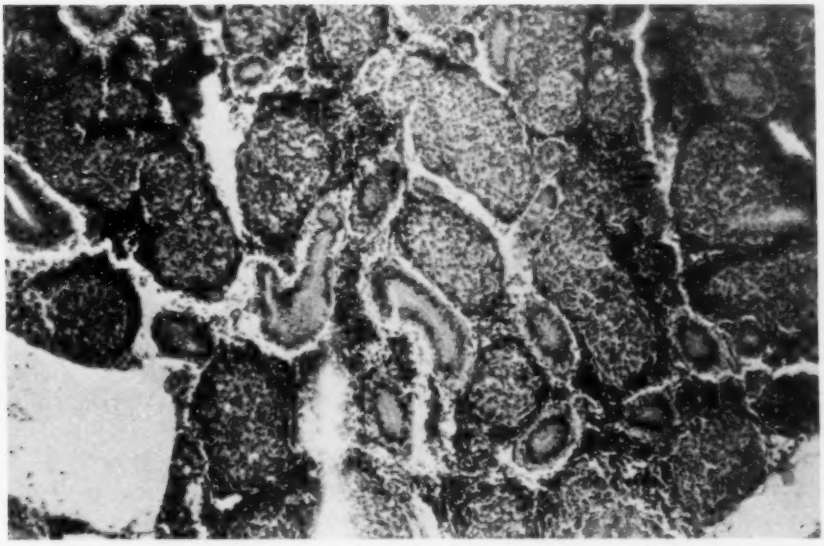

FIG. 4. Kidney at 12 months, showing displacement of closely aggregated tubules by massive accumulations of tumor cells. (× 6.5)

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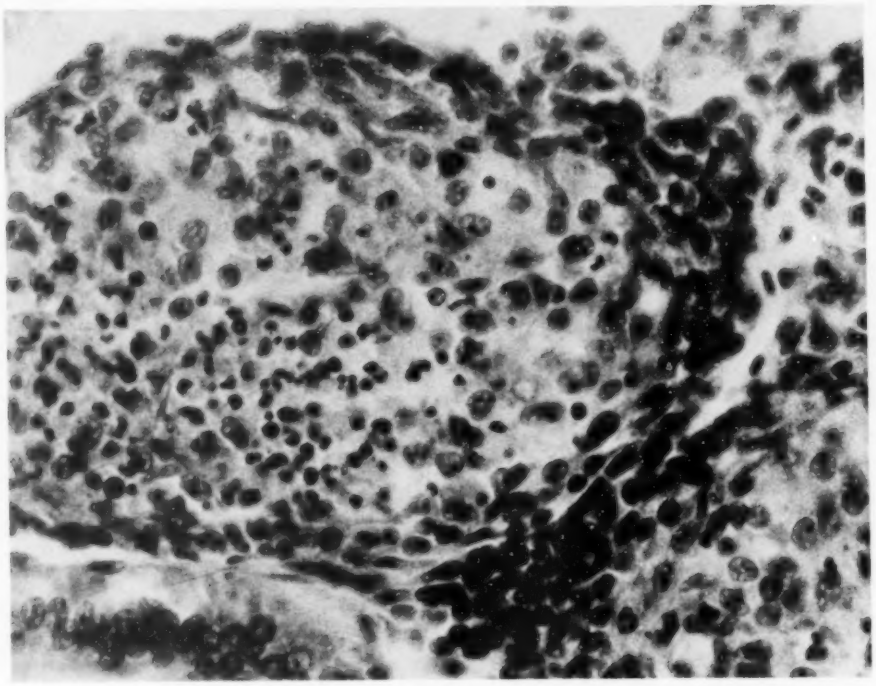

FIG. 5. Tumor-cell mass from the kidney which is well circumscribed and delineated. Note the core of large cells with faintly staining nuclei as well as numerous pyknotic nuclei. (× 40)

DISCUSSION

In search of newer and more unique animal models to study aging, with special emphasis on the immune system, investigators are turning to invertebrates and to fish, amphibians, and reptiles (Cooper and Walford, 1982). Invertebrates and fish constitute the two major phylogenetic groups in which most of the work on comparative gerontology has been performed (Cooper, 1979). Fish as vertebrates, with simpler but similar cell and organ systems to humans thus provide models that can answer questions of direct bearing on the problem of senescence in humans. For example, the immune system of fish consists of leukocytes in the circulating blood, the thymus, spleen and the kidney, and it is known that fish are capable of both cellular and humoral immune responses (Cooper, 1976). Thus it is becoming generally accepted that fish possess the equivalent of functional T and B lymphocytes, an immune system essentially like that of mammals (Van Muiswinkel and Cooper, 1981).

During the early 1960s, Walford (1964) advanced his immunologic theory of aging, suggesting that the existence of age-related immunologic disharmony was probably autoimmune in nature. According to this view, immunologic responses represent the cause of aging in contrast to the view of those who propose that changes in the immune system are a consequence of aging. In both instances, however, the degree of immunologic competence at various ages in the life cycle are related to overall physiologic homeostasis, and

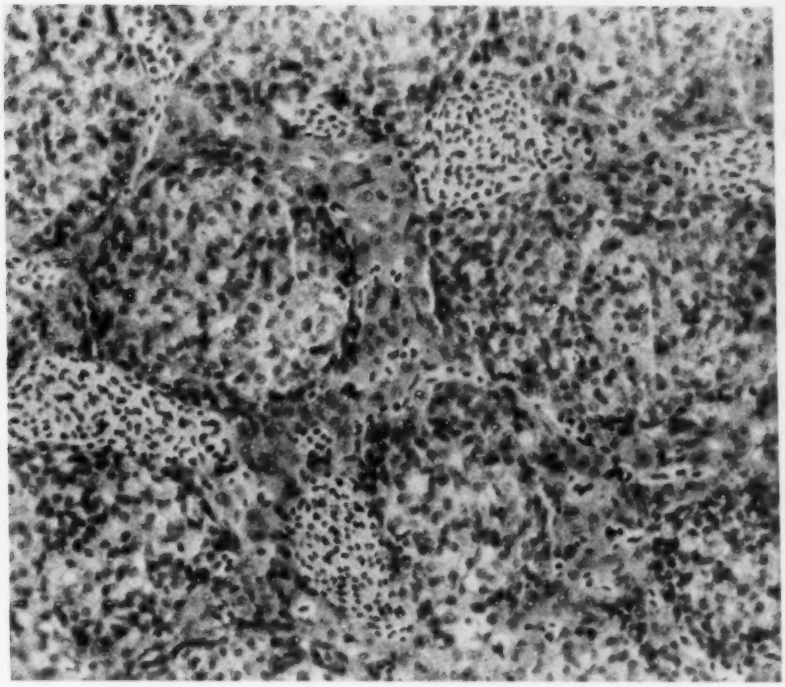

FIG. 6. The liver showing only a few cords of normal hepatocytes. Otherwise the entire organ consists of tumors which are well circumscribed masses. (× 16)

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any changes or disharmony will certainly affect adaptation and survival (Timiras, 1978). In support of the second view, Kay (1979) has stressed that certain immune functions tend normally to decline with age in at least six mammalian species and in humans. Furthermore, she has shown that as immunologic vigor decreases, the incidence of auto-immune immune-complex diseases and cancer increases. Emphasizing one aspect of the immune system (that mediated by T lymphocytes), she presents earlier evidence that restoration to normality (or reversal of these deficiencies) can be accomplished by reconstituting immunologically deficient animals with young, but not old, syngeneic thymus or spleen grafts (Fabris, 1977; Piantanelli and Fabris, 1977; Kay, 1979). Thus, the evidence is clear that in mammals, T-lymphocyte function declines with age.

In this present work, we have pointed out for the first time the marked senescent changes that occur in the thymus of fish. These histopathologic changes are strikingly similar to those that occur in mammals and that have been reported in another teleost, the mouth breeder (Sailendri, 1973) and in an amphibian, the bullfrog (Cooper, 1967). The unique feature of our histopathologic analysis in *N. guentheri* and *C. adloffi* is that as the thymus undergoes senescence, there is an increase in all fish in age-associated pathologic changes such as cancer at the maximum period of 12 months.

Thus we have extended the pioneer work by Walford and Liu (1965) on the histopathologic changes of organs other than the thymus. Moreover, we have confirmed

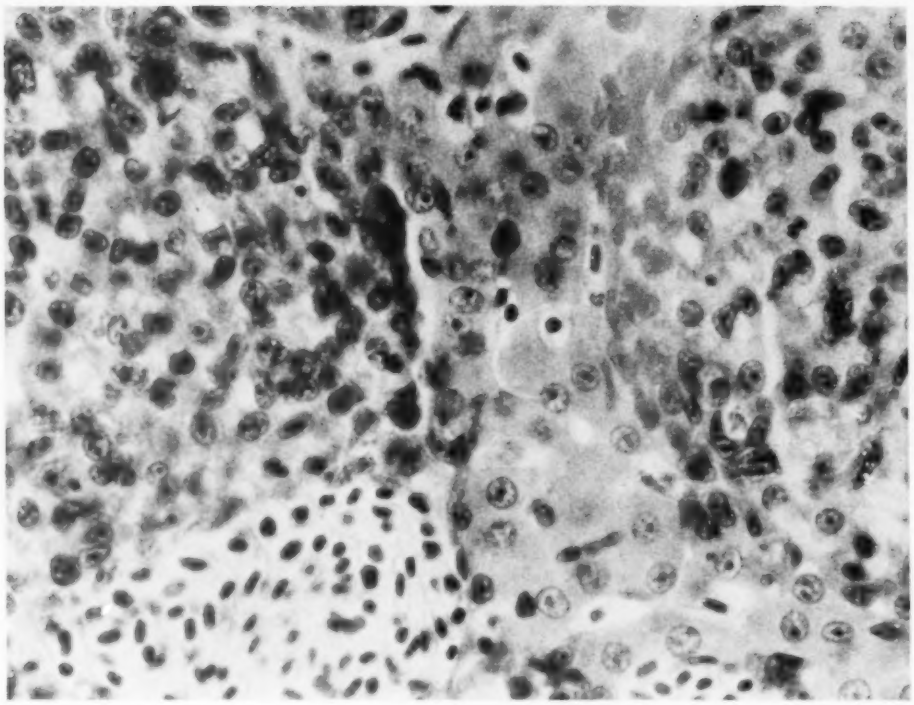

FIG. 7. A few normal hepatocytes are located in the center squeezed on both the left and right sides by well-circumscribed masses of tumor cells. (× 40)

the observations of Markofsky and Milstoc (1979a,b) that significant aspects of the senescent changes in the kidney and liver of *N. guentheri* involved malignant transformation where, in the kidney, it was referred to as nodular type *histiocytic lymphoma*. The cancer was characterized by increased numbers of large cells within a multicentric nodular aggregation. The cells were irregular in size and shape, with a pale pinkish, ill-defined cytoplasm which often contained lipochrome pigment. Often these cells were surrounded by small lymphocytes which, of course, showed greater cytoplasmic basophilia and pyknotic nuclei.

Since the thymus is the source of T lymphocytes in vertebrates, the obvious question remaining from our histopathologic analysis of thymus degeneration during senescence in fish is: how can these animals best serve as unique models for understanding the intrinsic or extrinsic causes of senescence? Obviously, there are two points that are clear. First, there is an increase in certain diseases as the immune system declines, and, obviously, there is an increase in life expectancy as in humans. Thus, understanding the aging immune system is of prime importance from the biological viewpoint since there are these analogies that are comparable. Fish are certainly useful in the more pragmatic sense for what they may offer relevant to clarifying senescence in humans. Second, fish may offer a reason unique to them which will be defined later. According to Kay (1979), future studies should be directed toward understanding the processes that regulate growth and atrophy

of the thymus, concentrating on the neuroendocrine thymic axis. In this regard, fish are unique, providing models for examining the role of seasons of the year, light and nutrition on immune function.

It is known, for example, that in the Japanese annual fish, the ayu and the ice-goby, the greatest volume of the thymus occurs in June and July (see review by Tamura, 1978). The thymus undergoes a remarkable involution prior to degeneration of the lymphoid organs, spleen and kidney (head, anterior or pronephros) at sexual maturation, and this was also observed in the mature chum salmon during its spawning migration. In other experiments, short photoperiods caused involution of the thymus which paralleled sexual maturation of the fish one to two months earlier than in control groups if the ayu were reared in outdoor hatchery ponds. In contrast, regressive changes in the thymus and sexual maturation of the ayu occurred during long photoperiods, but one to two months later than in controls. These represent but two examples of the pronounced relationship that must exist between the thymus and the neuroendocrine system. Fish will prove to be fascinating in dissecting these interactions, with more precision, in the future.

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SUMMARY

The thymus of aging annual, cyprinodont fish, *Nothobranchius guentheri* and *Cynolebias adloffi* begins to undergo senescent changes at approximately four months. At this time, the histopathology reveals increased amounts of connective tissue, epithelial cysts and myoid cells, but decreased numbers of thymocytes. At 12 months, of the 50 fish observed at 10 stages from day 15 and 1, 2, 4, 6, 8, 9, 10, 11, 12 months the maximum time of senescence, there was an increased incidence of tumors in the oral cavity, connective tissue, kidney and liver in all 5 fish. At least in the kidney, the malignant transformation was classified as nodular-type *histiocytic lymphoma*. It is of interest that development of these tumors occurs during peak senescent changes in the thymus, supporting the view that as fish age, accompanied by atrophy of the thymus, the immune system is less vigorous and there is an increase in the incidence of cancer.

Acknowledgments—We acknowledge Mr. R. Van Veen of Los Angeles who supplied the fish. This investigation was supported in part by research grants HD 09333-07 from NIH to ELC; Postdoctoral Grant, Education and Science Minister SPAIN (AZ); COFAA-IPN Grant, Mexico (JAR).

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THE EFFECT OF AGE ON SERUM PROTEINS IN MICE

JARED D. RODGERS[1] and GEORGE H. GASS[2]

(*Received* 22 *March* 1982)

Abstract—The relation of serum protein levels to age was investigated in the Swiss mouse. The present study demonstrates for the first time a dynamic age-dependent relationship between the various serum proteins. Total serum protein displayed a nonlinear change, decreasing from 10 to 136 days of age and thereafter increasing with advancing age with a peak at 465 days of age. Globulin concentration increased throughout the life span while albumin decreased for the first 136 days, after which it rose and peaked at 465 days of age. The relative percentage of each serum protein undergoes a significant non-constant, non-linear and non-quadratic change with increasing age. The patterns displayed by the various globulin fractions are strikingly similar to each other and tend to be inversely related to the pattern displayed by albumin. The relative percentage of each globulin fraction displayed a biphasic change with a peak at 200 days and a trough at 500 days of age.

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INTRODUCTION

ABSOLUTE AND RELATIVE changes in serum protein concentrations have been useful in elucidating disease processes. However, there is scant information and even less consensus as to age-dependent shifts which might affect the interpretation of these data. Past studies have not provided sufficient information to make meaningful and valid conclusions regarding alterations in serum protein levels throughout the life span (Finch and Forster, 1973; Amici, Granfranceschi, Maisili and Michetti, 1974; Salatka, Kresge, Harris, Edelstein and Ove, 1971; Coleman, Barthold, Osbaldiston, Foster and Jonas, 1977). The difficulties stem in part from comparisons of dissimilar age groups among animal strains having different life spans. More importantly, most studies have examined only a few age groups. The present communication investigates serum protein concentrations throughout the entire life span of the Swiss mouse.

MATERIALS AND METHODS

Male Swiss mice, raised at the breeding facility of the Endocrinologic Pharmacology Research Laboratory, Southern Illinois University, were maintained at 5 per cage at 23 ± 2° and approximately 50% humidity on a

[1]Present address and to whom all correspondence should be sent: Dr. Jared D. Rodgers, Department of Surgery, University of Alabama in Birmingham, Birmingham, Alabama 35294.

[2]Present address: Dr. George H. Gass, Chairman, Basic Science Department, Oklahoma College of Osteopathic Medicine and Surgery, P.O. Box 2280, Tulsa, Oklahoma 74101.

12:12 light-dark cycle. All litters were reduced to 8 pups at time of birth. The mice were weaned at 18 days of age and fed (Purina Laboratory Chow) and watered *ad libitum*. The mice were randomly selected from the colony and the ages ranged from 10 to 663 days. All mice in an age group were of the same age. The maximum life span of the males in this colony was 720 days.

Blood was procured by orbital bleeding into non-heparinized hematocrit tubes. Serum protein was determined by the method of Lowry, Rosebrough, Farr, and Randolph (1951), using bovine serum albumin as the standard. Electrophoresis of the serum was performed on cellulose strips at 270 V for 60 minutes in 0.05M Tris-barbital buffer (Gelman Instrument Co., MI), pH 8.8 (Briere and Mull, 1964). The strips were stained in 3% TCA containing 0.2% Ponceau S for 10 minutes. Destaining was performed with 3 one-minute rinses in 5% acetic acid, a one-minute rinse in absolute methanol and a three-minute rinse in 15% acetic methanol. The strips were scanned by a densitometer (Densicord) and the relative amounts of the individual serum proteins were quantitated by measuring the areas under the curves.

Statistical analysis

Data points for total serum protein, absolute amount and relative percentage of each serum protein fraction throughout the lifespan were fit to the following regression equation: Serum Protein = $B_0 + B_1\text{Days} + B_2\text{Days}^2 + B_3\text{Days}^3$. The third order or cubic term (B_3) was utilized to test the likelihood that some patterns of change with age did not remain constant with age, did not change linearly with age and did not change according to a simple quadratic law. Determinations of the level of significance were made for the cubic, quadratic and linear terms for each set of data. If the third order term in the regression equation was significantly different from 0 ($p < 0.05$), then the cubic term was required and the linear and quadratic models were not valid. Curves were generated from the appropriate model to fit the data points throughout the lifespan. The means of total serum protein concentration for each age group were tested with Duncan's Multiple Range Test at $p = .05$.

RESULTS

Total serum protein concentration decreases from 6.3 g/dl at 10 days of age to 4.9 g/dl at 136 days of age and then gradually increases and peaks at 11.4 g/dl at 465 days of age (Fig. 1). The protein concentration means on days 329, 465 and 663 are significantly greater than on day 10 ($p = .05$). The data were fit to the aforementioned regression model, which produces the following equation: Total serum protein = $5.69 - 0.01\text{Days} + 0.09 \times 10^{-3}\text{Days}^2 - 0.10 \times 10^{-6}\text{Days}^3$. Since the cubic coefficient is significantly different from zero ($p = .0007$), the cubic model is required.

Cellulose acetate electrophoresis of mouse serum results in the separation of 7 protein bands. Two lightly staining protein bands, found between the alpha 2 and beta bands, are designated as post-alpha and pre-beta proteins. The mean concentration (g/dl) of each serum protein fraction throughout the lifespan is given in Table 1. The cubic model (cubic term, $p = .0001$) is required for albumin. The predicted curve is biphasic with a trough at 120 days of age and a peak at 500 days of age. Alpha 1, beta and gamma globulins increases linearly with age (linear term, $p = .0001$, cubic and quadratic terms not significant), while alpha 2 globulin displays a biphasic curve (cubic term $p = .0044$) increasing at the early and late ages.

The regression for the relative percentages of the major serum protein fractions across age produces the following equations:

$$\begin{aligned}
\text{Albumin} &= 72.5 - 0.24\text{Days} + 9.2 \times 10^{-4}\text{Days}^2 - 9.4 \times 10^{-7}\text{Days}^3 \\
\text{Alpha 1} &= 6.0 + 0.05\text{Days} - 2.1 \times 10^{-4}\text{Days}^2 + 2.2 \times 10^{-7}\text{Days}^3 \\
\text{Alpha 2} &= 5.5 + 0.09\text{Days} - 3.3 \times 10^{-4}\text{Days}^2 + 3.3 \times 10^{-7}\text{Days}^3 \\
\text{Beta} &= 10.4 + 0.06\text{Days} - 2.2 \times 10^{-4}\text{Days}^2 + 2.3 \times 10^{-7}\text{Days}^3 \\
\text{Gamma} &= 5.6 + 0.04\text{Days} - 1.6 \times 10^{-4}\text{Days}^2 + 1.6 \times 10^{-7}\text{Days}^3
\end{aligned}$$

The cubic term of each regression equation is significant to $p = .0001$. The predicted

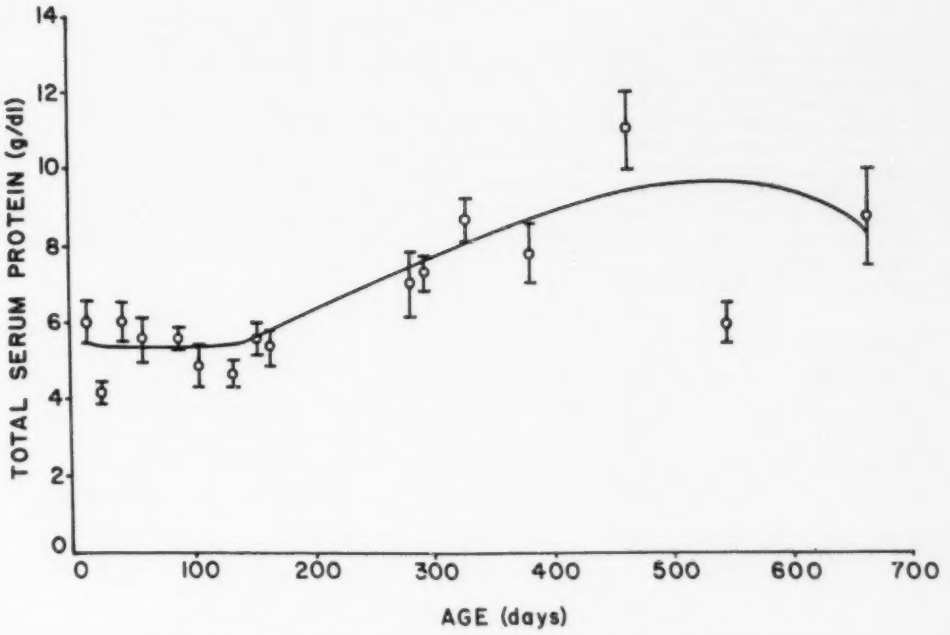

FIG. 1. The relation of total serum protein concentration to advancing age. Vertical bars represent standard error of the mean. The data points were fit to a regression equation and the solid line represents the curve of best fit. The number of mice used per data point is indicated in Table 1.

curve (solid line) for each globulin fraction is biphasic with a peak at approximately 200 days of age and a trough at approximately 500 days of age. These curves are inversely related to the predicted curve for albumin (Fig. 2).

DISCUSSION

Total and individual serum proteins have been reported to increase, decrease or undergo no significant change with advancing age (Finch and Forster, 1973; Amici *et al.*, 1974; Salatka *et al.*, 1971; Coleman *et al.*, 1977). This lack of consensus is due primarily to the small number of age groups compared. The results of our study, based upon sixteen age groups encompassing the entire life span of our Swiss mouse colony, indicate that the pattern of total serum protein concentration is curvilinear as a function of age (Fig. 1). There is a downward trend from 42–136 days and an upward trend thereafter with a peak at 467 days of age. The downward trend occurs during rapid growth of the mice (Crispens, 1975); beyond 136 days of age the changes occur during maturation and senescence. The low serum protein value at 21 days of age coincides with a loss in body weight following the abrupt change in diet at weaning. It is not clear why at 548 days of age the serum protein is much lower than the predicted curve. On visual inspection these mice appeared to be healthy, but nevertheless might be subject to an unseen infection or disease process which might have affected the results.

Cellulose acetate electrophoresis of serum results in the separation of two previously unreported bands which together account for approximately 3% of the serum protein. On

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TABLE 1. MEAN ± SE VALUES FOR SERUM PROTEIN CONCENTRATIONS (G/DL) IN DIFFERENT AGED MICE

Age (days)	Albumin	Alpha 1	Alpha 2	Post-Alpha	Pre-Beta	Beta	Gamma
10 (10)	4.39 ± 0.38	0.29 ± 0.04	0.21 ± 0.02	0.06 ± 0.01	0.10 ± 0.02	0.86 ± 0.08	0.41 ± 0.04
21 (10)	2.97 ± 0.17	0.35 ± 0.05	0.23 ± 0.03	0.06 ± 0.01	0.03 ± 0.01	0.39 ± 0.06	0.25 ± 0.04
42 (15)	4.22 ± 0.33	0.37 ± 0.03	0.55 ± 0.06	0.14 ± 0.01	0.16 ± 0.03	0.67 ± 0.05	0.31 ± 0.03
56 (9)	2.95 ± 0.31	0.59 ± 0.08	0.73 ± 0.09	0.15 ± 0.04	0.12 ± 0.03	0.71 ± 0.11	0.51 ± 0.01
86 (7)	2.98 ± 0.15	0.60 ± 0.06	0.71 ± 0.05	0.15 ± 0.06	0.03 ± 0.01	0.67 ± 0.04	0.60 ± 0.07
107 (10)	2.48 ± 0.29	0.47 ± 0.06	0.72 ± 0.09	0.19 ± 0.03	0.08 ± 0.02	0.87 ± 0.08	0.49 ± 0.04
136 (9)	2.42 ± 0.17	0.58 ± 0.04	0.55 ± 0.03	0.14 ± 0.01	0.03 ± 0.02	0.71 ± 0.07	0.43 ± 0.03
153 (9)	3.06 ± 0.27	0.63 ± 0.04	0.66 ± 0.08	0.10 ± 0.02	0.03 ± 0.01	0.76 ± 0.06	0.53 ± 0.06
167 (14)	2.93 ± 0.23	0.55 ± 0.06	0.68 ± 0.08	0.15 ± 0.03	0.04 ± 0.01	0.81 ± 0.05	0.52 ± 0.06
285 (5)	4.50 ± 0.47	0.53 ± 0.09	0.68 ± 0.07	0.14 ± 0.04	0.09 ± 0.04	0.84 ± 0.16	0.49 ± 0.14
296 (15)	4.65 ± 0.34	0.58 ± 0.06	0.64 ± 0.05	0.12 ± 0.02	0.08 ± 0.03	0.83 ± 0.03	0.56 ± 0.06
329 (23)	5.07 ± 0.29	0.68 ± 0.07	0.85 ± 0.05	0.21 ± 0.03	0.05 ± 0.01	1.27 ± 0.12	0.71 ± 0.09
382 (10)	5.02 ± 0.49	0.45 ± 0.06	0.60 ± 0.10	0.11 ± 0.03	0.06 ± 0.02	1.03 ± 0.12	0.73 ± 0.10
465 (9)	6.89 ± 0.67	1.09 ± 0.11	1.05 ± 0.11	0.21 ± 0.02	0.15 ± 0.03	1.17 ± 0.11	0.79 ± 0.08
548 (5)	3.16 ± 0.27	0.56 ± 0.06	0.85 ± 0.13	0.19 ± 0.04	0.11 ± 0.08	0.81 ± 0.06	0.52 ± 0.03
663 (7)	4.29 ± 0.42	0.84 ± 0.12	1.19 ± 0.28	0.16 ± 0.05	0.07 ± 0.03	1.59 ± 0.29	0.81 ± 0.15

Parentheses indicates the number of mice. Post-alpha and pre-beta bands are described in the text.

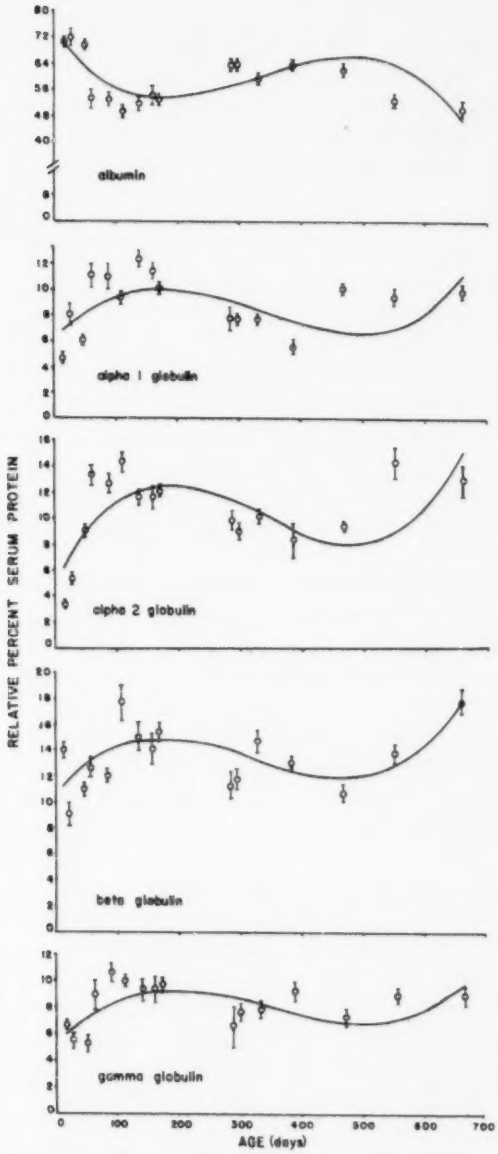

FIG. 2. The relation of relative percentages of individual serum proteins to advancing age. Vertical bars represent standard error of the mean. The data points were fit to a regression equation and the solid line represents the curve of best fit.

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the basis of their migrational pattern, they were designated post-alpha and pre-beta bands. It is possible that these bands represent the additional electrophoretic fractions of the beta and alpha 2 proteins that have been separated by starch gel electrophoresis (Daams, 1964). When the concentration of each serum protein across age is tested by the cubic model, albumin and alpha 2 globulin exhibit biphasic changes, while alpha 1, beta and gamma globulins increase linearly. Albumin concentration, which decreases for the first 136 days and peaks at 465 days of age, exhibits a pattern similar to that displayed by total serum protein, while alpha 2 globulin increases early in life, plateaus, then increases further in later life (Table 1).

With regard to the relative percentages of the serum proteins, most studies agree that with advancing age globulins increase (Salatka *et al.*, 1971). This increase in the globulin fraction has been variously attributed to alpha (Salatka *et al.*, 1971; Coleman *et al.*, 1977), gamma (Quinn, Price, Ellis and Makinodan, 1973) or even total globulins (Salatka, *et al.*, 1971). The present study demonstrates for the first time a dynamic age-dependent relationship between the various serum proteins. The relative percentage of each serum protein undergoes a significant nonconstant, nonlinear and non-quadratic change with increasing age. The patterns displayed by the various globulin fractions are strikingly similar to each other and tend to be inversely related to the pattern displayed by albumin (Fig. 2).

A unilateral inverse relationship between albumin and globulin proteins in disease states was initially described by Wuhrmann and Wunderly (1960). Later studies showed that elevated levels of gamma globulin caused a corresponding decrease in both the absolute and relative amounts of albumin, presumably so as to maintain the oncotic pressure (Bjorneboe, 1967; Rothschild, Oratz, Franklin and Schreiber, 1967). Further research indicated that other agents capable of increasing the intravascular oncotic pressure or colloid content of hepatic interstitial fluid were also competent to decrease the rate of albumin synthesis, whereas the gamma globulin level appeared to be independent of oncotic control (Oratz, Rothschild and Schreiber, 1977). This apparent colloid osmotic pressure regulation of the albumin level could explain the observed decrease in the absolute and relative amounts of albumin which accompanies the increase in globulins in young mice. In older mice, however, the level of albumin increases despite the continued rise in globulin concentration. The increase is consistent with the reported increased synthesis of albumin in aged rats (Obenrader, Chen, Ove and Lansing, 1974) and in liver parenchymal cells isolated from 36 months old rats (van Bezooijen, Grell and Knook, 1976). Consequently, there are probably many factors (nutrition, infection, vascular volume, hormonal changes, etc.) besides colloid osmotic pressure that contribute to the changing levels of the serum proteins throughout the life span of the mice.

SUMMARY

The relation of serum protein levels to age was investigated in the Swiss mouse. Total serum protein displayed a nonlinear change, decreasing from 10 to 136 days of age and thereafter increasing with advancing age with a peak at 465 days of age. Cellulose acetate electrophoresis of serum resulted in the separation of two previously unreported bands, presumably related to the alpha 2 and beta fractions, which accounted for 3% of the total serum protein. Globulin concentration increased throughout the life span while albumin decreased for the first 136 days, after which it rose and peaked at 465 days of age. The

relative percentage of each globulin fraction displayed a biphasic change with a peak at 200 days and a trough at 500 days of age. The relative percentage of albumin was inversely related to these changes.

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A LONGITUDINAL STUDY OF THE RELATIONSHIP BETWEEN AGING AND THE DURATION OF HEXOBARBITAL HYPNOSIS IN MALE CFN RATS

MALCOLM B. BAIRD

Masonic Medical Research Laboratory, 2150 Bleecker Street, Utica, New York 13503

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Abstract—The duration of hexobarbital hypnosis was determined for individual male CFN rats at three month intervals over their total adult lifespan. The mean duration of hexobarbital hypnosis for the cohort increased linearly with advancing age. However, the sleeptimes for *individual* rats fell into one of four categories (gradual increase, terminal step increase, no change, or not possible to assign) when assigned as a function of duration of response to the barbiturate over the lifespan of the animals.

These results suggest that the generally well-accepted increase in duration of drug response in aging animals is neither a ubiquitous nor concomitant feature of aging. Furthermore, the varying results obtained for individual animals in this longitudinal study point out the possible error which may be attendant when utilizing pooled tissue extracts to examine hepatic monooxygenase activity *in vitro* in senescent rodents.

INTRODUCTION

THE MICROSOMAL mixed-function oxidases are a complex of enzymic functions which normally carry out the intermediary metabolism of steroids and fatty acids (Conney, 1967). These functions also accept a wide variety of drugs, pesticides, carcinogens and other potentially toxic compounds (e.g., naturally occurring compounds such as aflatoxins) as substrates.

There is considerable interest in a critical examination of the integrity of the microsomal mixed-function oxidases in senescent organisms for several reasons: 1) A disproportionate consumption of drugs, as well as of frequency of adverse drug reactions, are manifest in the elderly segment of the human population (Bender, 1974; Richey and Bender, 1977). 2) Age-related changes in the metabolism of toxic or potentially toxic compounds, to which all members of the human population are exposed, may place the elderly segment of the population at increased risk to the untoward effects associated with these compounds. Since some human tumors certainly result from association with chemical carcinogens, an important corollary of this suggestion is that age-associated changes in this enzyme system may be, in part, responsible for the well known age-related increase in tumor incidence and mortality for many forms of cancer (Baird, Birnbaum and Massie, 1976; Baird and Birnbaum, 1979). 3) Finally, an exhaustive analysis of this enzyme system in a variety of organs within aging animals would lead to a better understanding of both

the integrity and organization of cellular membranes in senescent organisms in a model system which is currently under intense scrutiny in many laboratories.

Unfortunately, interest in studies concerning the integrity and functional capacity of the mixed-function oxidases in senescent organisms has been somewhat dampened by the often conflicting, and therefore confusing, reports concerning the effect(s) of aging on the activities of the varied hepatic monooxygenase enzyme functions, the levels of the macromolecular components which specify those activities, and of the regulatory control capacity of this enzyme system as measured by response to exogenous inducers.

Various attempts have been made to assess the functional capacity of the hepatic microsomal mixed-function oxidases within intact aging rodents. These studies generally examined the duration of action of a variety of pharmacological agents which require metabolic activation or detoxification mediated by the hepatic monooxygenases in animals (including humans) of varying chronological age (Streicher and Garbus, 1955; Kato and Takanaka, 1968a; Baird, Samis and Massie, 1971; O'Malley, Crooks, Duke and Stevenson, 1971; Jori, DiSalle and Quadri, 1972; Baird, Nicolosi, Massie and Samis, 1975; Baird, Zimmerman, Massie and Pacilio, 1976). This approach seems attractive in that it is thought to be possible to subsequently correlate age-associated changes in response to various xenobiotics *in vivo* with age-related changes in the capacity of senescent animals to metabolize these compounds *in vitro*.

All such studies to date have been cross-sectional in nature, and suggest, in general, that the mean duration of drug response increases with advancing age for those drugs which are detoxified by the hepatic monooxygenases. However, our studies show that although the duration of drug action generally increases with advancing age, there is considerable overlap in the data, with many rodents actually showing no age-related increase in the duration of action of either hexobarbital or of the muscle relaxant zoxaxolamine (Baird, Samis and Massie, 1971; Baird, Nicolosi, Massie and Samis, 1975; Baird, Zimmerman, Massie and Pacilio, 1976 and unpublished data for the duration of hexobarbital hypnosis in C57BL/6J male mice of varying ages).

It occurred to us that longitudinal studies, in which the response of individual animals to pharmacological agents would be determined several times over the lifespan of the animal, may be more useful than cross-sectional studies in attempting to assess the functional integrity of the hepatic mixed-function oxidase system in aging rodents since there would be no selection for surviving, distinct subpopulations of animals as may occur in cross-sectional studies. The present study reports the duration of hexobarbital hypnosis in male CFN rats determined at regular intervals over the duration of their adult lifespan.

MATERIALS AND METHODS

Male CFN rats (Wistar-derived) were used in this study. The animals were produced in our Laboratory, weaned at 21 days of age and maintained in an aging colony under environmental conditions described previously (Baird, Nicolosi, Massie and Samis, 1975).

Duration of hexobarbital hypnosis was determined essentially as described elsewhere (Baird, Nicolosi, Massie and Samis, 1975) for each surviving rat at three month intervals over the life time of the animal. Rats were injected intraperitoneally with sodium hexobarbital (40 mg/ml in 0.9% NaCl) at a dose level of 100 mg/Kg body weight. Injections were always made at 0900–0915 EST. Sleeping rats were placed on their backs in a quiet, draft-free room and subjected both to an auditory stimulus (three-second bell alarm) and to a physical stimulus (pinched ear) at two minute intervals. The time required to recover the "righting reflex" was recorded for each animal. Surviving animals were immediately returned to the aging colony at the conclusion of each test.

Sodium hexobarbital was purchased in sealed glass ampules from Sterling-Winthrop, Inc., Rensselaer, NY. Hexobarbital solutions were prepared immediately prior to use and purged of dissolved gases by bubbling with

helium. Samples were diluted 1:1000 with distilled water and the absorption spectrum of each solution recorded with a Varian 118C spectrophotometer immediately before and immediately following the conclusion of the injections. Reaction of hexobarbital with atmospheric carbon dioxide, as determined by comparing absorbance at 344 nm prior to and immediately following injection was negligible over the 15 minutes of time required to inject the animals.

RESULTS

The mean duration of hexobarbital hypnosis increased with advancing age in CFN male rats (Table 1). This increase was linearly dependent upon advancing age when mean sleep-times for the cohort were plotted (Fig. 1). Greatly increased unpredictability in mean sleep-

TABLE 1. DURATION OF HEXOBARBITAL HYPNOSIS (MINUTES) FOR INDIVIDUAL CFN MALE RATS TESTED AT THREE MONTH INTERVALS THROUGHOUT THEIR ADULT LIFESPAN.

Animal No.	*age in months*								
	4	7	10	13	16	19	22	25	28
1	19	27	18	*	24	109	†		
2	*	29	37	27	32	67	55	82	†
3	23	26	32	31	20	24	77	†	
4	20	23	26	31	45	50	56	**	
5	13	15	19	14	39	20	46	54	†
6	13	16	25	†					
7	16	22	23	21	41	36	38	57	†
8	19	17	20	27	†				
9	20	25	28	40	51	**			
10	22	18	25	32	47	57	35	†	
11	16	††	25	60	42	46	††		
12	22	25	37	54	44	54	†		
13	18	25	24	21	26	39	38	**	
14	21	25	33	44	50	†			
15	14	22	16	21	31	22	18	40	†
16	27	*	35	40	46	55	59	68	†
17	23	24	19	23	26	*	26	113	56
18	22	28	33	36	36	36	†		
19	23	40	29	52	50	49	49	†	
20	25	28	54	46	169	>360	†		
21	**	20	26	31	30	*	37	45	36
22	20	29	25	24	33	56	76	†	
23	15	24	27	30	17	†			
24	14	28	28	32	†				
25	12	19	19	22	30	33	**		
26	18	21	42	58	32	38	58	44	49
27	15	21	18	33	24	62	25	**	
28	19	30	22	22	32	**			
29	*	23	18	45	27	48	43	53	**
$\bar{x}$	19	24	27	34	40	47	45	62	47

*no hypnosis following hexobarbital injection

**died following hexobarbital injection

†died during experiment at times other than following hexobarbital injection

††improperly dosed with hexobarbital

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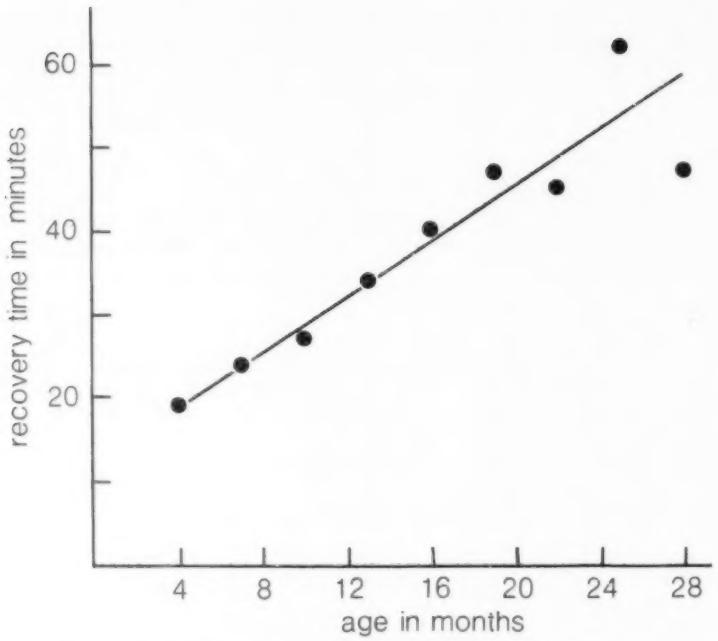

FIG. 1. Effect of advancing age upon mean duration of hexobarbital hypnosis for a cohort of male CFN rats.

times was observed when survivors in the cohort were greater than twenty months of age (Fig. 1), which we attribute to an age-related increase in the number of animals which die following hexobarbital injection (Table 1). These animals have, in effect, an infinitely long recovery time but cannot be included in the mean values for the duration of barbiturate hypnosis in any meaningful way. Thus, mean recovery time from hexobarbital hypnosis in senescent CFN rats that survive the injection may be artifactually low.

Inspection of the duration of hexobarbital hypnosis for individual animals reveals large interanimal variability in drug response. Thus, there was no consistent or significant increase in duration of drug action for animals number 8,15,23 and 28. However, a gradual increase reminiscent of the mean values (Table 1 and Fig. 1) was observed for some animals (e.g., 2,4 and 5). In yet other animals there was little or no change in the duration of hexobarbital hypnosis for many months over the lifetime of the rats, followed by a terminal step increase in recovery time (e.g., animal no. 17).

We attempted to categorically assign each animal to the above mentioned four classes based on the individual response to hexobarbital over the duration of its lifespan. This assignment was made by two individuals not associated with this study because of the obvious possibility of bias in making the assignment. Approximately 70% of CFN rats generally exhibit an increase in the duration of hexobarbital hypnosis with advancing age (Table 2). This increase may be gradual (44.8% of all animals) or involve a terminal increase (25.9% of the rats). However, these age-associated changes seem not to be a ubiquitous feature of aging in rats, since it does not occur in all animals (e.g., animal no. 15), nor is it possible to assign some animals to any group (e.g., no. 27). This finding

TABLE 2. ASSIGNMENT OF INDIVIDUAL MALE CFN RATS TO FOUR CATEGORIES RELATING TO THE EFFECT OF AGING ON DURATION OF HEXOBARBITAL HYPNOSIS AS DETERMINED FOR INDIVIDUAL ANIMALS OVER THEIR ENTIRE LIFESPAN.*

	assignment 1	assignment 2	avg.	%
No Change	4	4	4	13.8
Gradual Increase	14	12	13	44.8
Step Increase	7	8	7.5	25.9
Cannot Tell	4	5	4.5	15.5
Total	29	29	29	100.0

*Assignment was carried out by two separate individuals (1 and 2).

demonstrates an inherent variability in the effect(s) of aging on this enzyme system, and suggests that an age-related increase in the duraton of action for at least one barbiturate may not be a concomitant feature of the aging process in rats.

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DISCUSSION

Streicher and Garbus (1955) originally reported a terminal increase in the duration of hexobarbital hypnosis in Sprague-Dawley male rats and a terminal decrease in females. These changes were attributed to age-related alterations in steroid hormone levels. Additionally, duration of hypnosis was prolonged at any age by treatment with SKF-525A, an inhibitor of hepatic mixed-function oxidase activity, or by administration of chloropromazine, a pharmacological agent which increases the sensitivity of the central nervous sytem to the action of barbiturates.

Kato and Takanaka (1968a) subsequently reported an increase in the duration of action of pentobarbital, carisoprodol and zoxazolamine in 600 day old male or female rats as compared to animals 100 days of age. Furthermore, these workers showed that the increase in duration of action of pentobarbital or carisoprodol paralleled an age-associated decline in the hepatic metabolism *in vitro* of these compounds. Finally there was no evidence for age-related differences in the sensitivity of the central nervous system to these compounds, and recent studies have shown that there is no apparent age-related difference in the number of narcotic receptors in the brains of C57BL/6J male mice (Webster, Shuster and Eleftheriou, 1976).

Work from our laboratory confirmed that the duration of both zoxazolamine paralysis in C57BL/6J male mice (Baird, Samis and Massie, 1971) and hexobarbital hypnosis in rats (Baird, Nicolosi, Massie and Samis, 1975; Baird, Zimmerman, Massie and Pacilio, 1976) increased with advancing age, and was paralleled by a decline in the *in vitro* metabolism of some xenobiotics by the hepatic microsomal enzyme system (Kato and Takanaka, 1968b,c; Baird, Samis and Massie, 1971; Baird, Nicolosi, Massie and Samis, 1975; Baird, Zimmerman, Massie and Pacilio, 1976; Birnbaum and Baird, 1978a,b; Birnbaum and Baird, 1979). However, our data also clearly showed considerable overlap among animals of varying age, in that some old animals exhibited drug-induced sleeptimes or duration of paralysis identical to that seen in their younger counterparts (Baird, Samis and Massie, 1971; Baird, Nicolosi, Massie and Samis, 1975; Baird, Zimmerman,

Massie and Pacilio, 1976). Thus, the often reported age-related increase in duration of drug action in senescent rodents appear not to reflect changes inextricably associated with aging. In addition, age-associated changes in hepatic drug metabolism appear to result from factors extrinsic to the liver (Baird, Zimmerman, Massie and Pacilio, 1976), are not a universal feature of aging, since they are not observed in ICR male mice (Kato, Takanaka and Onoda, 1970), and are reversible by treatment with various compounds (Gold and Widnell, 1974; Baird, Nicolosi, Massie and Samis, 1975; Birnbaum and Baird, 1978a,b; Kao and Hudson, 1980).

The present study clearly shows that there is, in general, an increase in the duration of hexobarbital hypnosis in male CFN rats with advancing age. However, the fact that substantial numbers of individual animals may not conform to this general statement suggests that examination of enzymic activity of hepatic monooxygenases in pooled extracts of hepatic microsomal membranes prepared from old rodents may be of somewhat limited value in understanding the integrity of this enzyme system in aging rodents. Use of pooled extracts may be one factor contributing to the considerable confusion in the literature, even among animals from identical strains, concerning the effects of aging on both the components of the hepatic monooxygenases and upon the enzymic functions which they specify (e.g. Kao and Hudson, 1980; Smucker and Wang, 1980; Rikans and Notley, 1981). However, conflicting results probably also arise from interlaboratory differences in the care of aging rodent colonies, as well as to differences in experimental design. Thus, comparisons of hepatic microsomal monooxygenases in sexually immature 1 month old rats with those of 14 or 15 month animals (Smucker and Wang, 1980) will certainly yield results which differ from comparisons of sexually mature 2 or 5 month old animals to their 14 or 15 month-old counterparts (Kao and Hudson, 1980; Rikans and Notley, 1981), since the functional capacity of this enzyme system seems to depend upon endogenous hormone levels (El Defrawy el Masry, Cohen and Mannering, 1974). Thus, some of the confusion in the literature could be eliminated by a modest attempt to compare animals of the same chronological age as those previously examined and reported by other workers.

Our results suggest that more clear and useful findings can be obtained by utilizing individual animals in studies of the relationship between aging and the functional capacity of the hepatic microsomal system. It is of obvious importance to continue to increase our understanding of the role of aging on the metabolism of pharmacological agents. This work gains additional importance in light of recent suggestions that age-associated changes in the metabolism of chemical carcinogens by microsomal enzyme systems may be one factor responsible for the well-known increased incidence of cancer with advancing age (Baird, Birnbaum and Massie, 1976; Birnbaum and Baird, 1979; Baird and Birnbaum, 1979).

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THE ADRENOCORTICAL STRESS-RESPONSE IN THE AGED MALE RAT: IMPAIRMENT OF RECOVERY FROM STRESS

ROBERT M. SAPOLSKY, LEWIS C. KREY[1] and BRUCE S. MCEWEN

The Rockefeller University, New York, N.Y. 10021

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Abstract—To test if the adrenocortical axis of the rat loses sensitivity to negative feedback control during aging, we have examined corticosterone secretion under basal, stressed and post-stress conditions in young and in aged Fisher male rats. We find no age-related change in the speed or magnitude of the stress response or in the capacity to manifest a corticosterone response following chronic stress. However, we do observe in aging rats an elevation of basal corticosterone and an impaired capacity to adapt to and recover from stress. This latter finding is illustrated by longer latencies relative to young rats, in the return of serum corticosterone concentrations to basal values during sustained exposure to mild cold or following the end of immobilization stress. All of these deficits reflect an increased rate of corticosterone secretion during physiologically inappropriate circumstances. Such observations support the concept that there is an age-related loss of sensitivity of the brain and pituitary to the inhibitory effects of high circulating levels of corticosterone on ACTH release.

Key words—Aging stress glucocorticoids adrenal cortex negative-feedback

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INTRODUCTION

SELYE AND TUCHWEBER (1976) have characterized aging as a systemic loss of adaptation. Numerous studies have examined this idea, and particular interest has focused on the function of the aging adrenocortical axis during stressed and non-stressed states. A number of authors (Riegle, 1976; Dilman, 1976) have postulated that the aging axis undergoes a decline in sensitivity to negative-feedback control. Such a view is based on the findings that aging rats display elevated basal corticosterone titers (Hoff, Scheff and Cotman, 1981; Tang and Phillips, 1978; Wexler, 1976; Landfield *et al.*, 1978 and 1979; Riegle and Hess, 1972 and 1973; Hess and Riegle, 1970 and 1972) and are somewhat resistant to the actions of the synthetic glucocorticod dexamethasone to suppress circulating corticosterone (Riegle and Hess, 1972). We have recently obtained additional support for this concept, in that the hippocampus, and to a lesser extent, the pituitary and amygdala, show unique depletions of nuclear corticosterone binding sites in the aged rat (Sapolsky, Krey and McEwen, 1983); these areas are frequently implicated in control of glucocorticoid negative-feedback. In an attempt to find additional aberrations

Please address all correspondence to: Bruce S. McEwen, The Rockefeller University, 1230 York Ave., New York, N.Y. 10021.

Reprint requests to: Robert M. Sapolsky, The Rockefeller University, 1230 York Ave., New York, N.Y. 10021.

[1]Alfred P. Sloan Foundation Fellow and Irma T. Hirschl Career Scientist

of adrenal function, we have re-examined corticosterone secretion in the aging rat under basal conditions and during response to and recovery from stress.

METHODS

Male Fisher 344 rats ranging from 3 to 27 months of age were provided by the Charles River Breeding Laboratory through a grant from the National Institute on Aging. Subjects arrived from a pathogen-free colony and were housed one/cage in isolated animal rooms for at least two weeks before use; mean lifespan is over 27 months in this colony. Subjects were maintained on a 12 h light: 12h dark schedule (lights on 0800) and were given food and water *ad libitum*.

Unless noted otherwise, blood samples in serum corticosterone determinations were collected from the tail vein. The subjects were immoblized in an animal restrainer and blood collected within two minutes after this procedure. Such a time-course has been validated as sufficiently rapid to provide basal corticosterone titers (Krieger, 1974). Fifty μl of blood were taken and centrifuged. Sera were seperated and frozen until assay. Corticosterone was measured by radioimmunoassay, according to the method of Krey, Lu, Hotchkiss, Piva and Knobil (1975) using an antiserum generated against cortisol 21-succinate bovine serum albumin (Antisera F21-53, Endocrine Sciences, Tarzana, CA). This antiserum cross-reacts with corticosterone (approximately 60%). In the present studies, 1,2,6,7 ^{3}H cortisol (Amersham) was used as the tracer and corticosterone as the standard. Assay sensitivity was 10 pg; coefficients of variation wtihin and between assays were 11% and 12%, respectively (n = 19).

Cage transfer stress

On the first day of the experiment at 0900, subjects were rapidly and quietly transferred to a new, clean cage in their home room. Blood samples were collected 30′ later and the rats were returned to their home cage. A second blood sample was taken the following day, at the same time as the initial sample. This latter sample represents basal levels.

Immobilization stress

At either 0900 or 2100, animals were rapidly transferred from their home cage to a restrainer which was adjusted so as to fully immobilize the rats. Blood was then collected within two minutes of restraint and then 15, 30 and 60 minutes later. Animals were then returned to their home cages.

Chronic stress

Subjects were stressed with one hour of immobilization, 2 minutes of exposure to ether, i.p. saline injection, one hour of rotation and vibration stress, and sustained cold (4°C) exposure. The rats received three of these stressors daily for a three-week period. All subjects received the same stressors in the same sequence; stressors were varied so as to minimize habituation to any single one. At the end of the three weeks, subjects were exposed to one hour of immobilization stress at 0900 and blood was collected at 1, 15, 30 and 60′.

Adaptation to moderate cold

Rats were anesthetized with ether between 1800 hours and 2000 h. A polyethylene catheter was inserted through the right jugular vein into the heart. This catheter, which exited through the skin between the shoulder blades, was filled with heparinized saline and then sealed. The next day, at 1000, the catheters were attached to another piece of tubing which led to a syringe situated outside the cage. An initial blood sample was taken within two minutes of this disturbance. The rats were then transferred into a room chilled to 4°C and left there for four hours. Blood samples were collected from the catheters at varying times during this period. This was done silently and out of view of the animals. A total of 50μl of blood was taken each time, and volume was replaced with a slow injection of saline. The catheters were removed at the end of the procedure under light anesthesia. Basal, non-stressed corticosterone determinations were obtained from the same subjects a number of weeks later.

Recovery from stress

Subjects were fitted with cardiac catheters and were given the one-hour immobilization stress described, at 0900. Animals were then returned to their home cages in their sound-insulated room and catheters were attached to syringes. Subjects were then bled through the catheters over the next 90 minutes to obtain samples of serum corticosterone.

Corticosterone clearance rate was determined by the method of De Souza and van Loon (1982). Briefly, subjects were fitted with cardiac catheters while under pentobarbital anesthesia (40 mg/kg body weight). One hour later, 5μCi of 1,2,6,7 ^{3}H corticosterone (Amersham, specific activity 82.1 Ci/nmole; suspended in 100μl of 1:1 saline:ethanol) was injected as a rapid pulse through the catheter. Blood samples (150μl) were taken immediately prior to injection and at 10 minute intervals up to the 50-minute mark. To replace the collected volume, saline was injected through the catheter. Blood was centrifuged and then 25μl of serum was extracted in duplicate with 400μl of methylene chlordide. The extract (300μl) was removed dried under vacuum and counted in Liquiscint (National Diagnostics, Somerville, NH, efficiency = 35%).

RESULTS

Male rats showed an age-related elevation in basal titers of corticosterone (Table 1). While this difference in basal levels with age was relatively small, similar differences were apparent in subsequent experiments (see time zero determinations in Table 2 and Figs. 1A, 1B and 3). Circadian rhythms in circulating corticosterone levels were present in old subjects (Table 1).

Cage transfer of rats caused a significant elevation in corticosterone levels after 30′ (Table 2). Although there was a trend towards higher basal and post-transfer corticosterone levels in aged subjects, this elevation was not statistically significant ($t = 1.55, 1.5$; $p < .10, .10$ for basal and post-transfer measures, respectively).

Figure 1 shows the magnitude and timecourse of the corticosterone response of young and aged rats to one hour of immobilization. Sections A and B show the responses of rats never before experimentally stressed, one hour into the light and dark cycles, respectively. Section C presents the stress-response one hour into light of rats previously exposed to three weeks of daily stressors. There was a significant elevation of corticosterone in each age group in response to each of the procedures (one-way ANOVA: initial stress in light, young, $f = 21$, old, $f = 21.4$; initial stress in dark, young, 10.5; old, 12.1; chronic stress, young, 7.4; old 7.6. All $p < .01$). Moreover, serum corticosterone responsiveness to immobilization showed a fluctuation over the course of the day; this is in agreement with studies showing a circardian rhythm of adrenal responsiveness to stimulation (Kaneko, K., Kaneko, M., Shinsoko and Dallman, 1981). Chronic stress caused a significant elevation of resting basal corticosterone levels in both age groups without changing the magnitude of the stress-response itself ($p < .05$, sign test). The critical point in these data is the lack of differences between the two age groups when either the absolute corticosterone titers or percentage increases above baseline were measured.

TABLE 1. BASAL CORTICOSTERONE TITERS OVER THE LIFESPAN OF THE MALE FISHER RAT (μg/100 ML)

Lighting Condition		Age (in months) 3	5	12	24	27
Light	$\bar{x}$	6.0	6.9	n.a.	10.4	11.5
(0800 hr)	S.E.	.75	1.9		1.0	1.5
	(n)	(8)	(5)		(8)	(5)
Dark	$\bar{x}$	12.6	n.a.	17.1	20.6	n.a.
(2000 hr)	S.E.	1.5		4.0	3.5	
	(n)	(8)		(6)	(10)	

n.a. = data not available. Blood was collected one hour into each lighting condition. See text for bleeding protocol. The increases at both times were significant at the .05 level (linear regression of age on corticosterone titer).

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TABLE 2. CORTICOSTERONE TITERS IN YOUNG AND OLD MALE FISHER RATS 30 MINUTES AFTER CAGE TRANSFER (μG/100 ML).

Age	Basal	Cage transfer	% rise
3 month (n = 12)	8.1 ± 1.6	15.2 ± 3.1	86
24 month (n = 13)	11.7 ± 2.6	21.2 ± 3.6	80

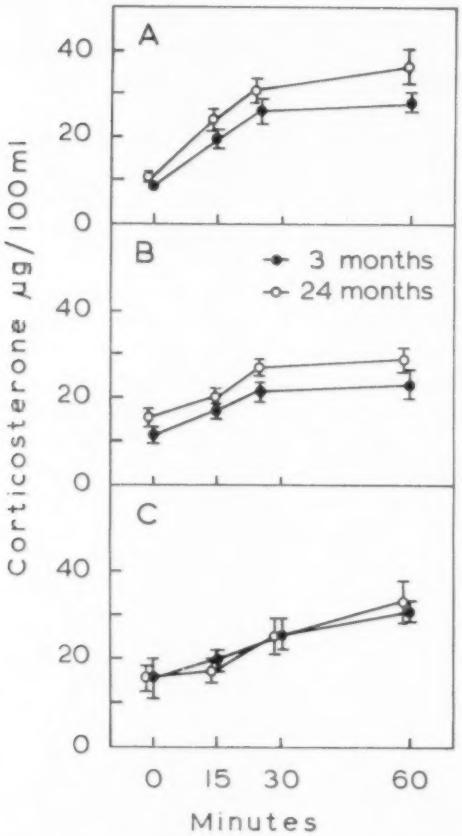

FIG. 1. Corticosterone titers in young and aged rats during 60 minutes of immobilization stress. Section A: Rats never before experimentally stressed, immobilized one hour into the light cycle. n = 20, 19 for young and aged, respectively. Section B: Rats never before experimentally stressed, immobilized one hour into the dark cycle. n = 11, 10 for young and aged, respectively. Section C: Rats subjected to three weeks of daily stress, immobilized one hour into the light cycle. n = 7, 7 for young and aged. Mean ± S.E.

The capacity to adapt to mild chronic stress in young and aged rats is presented in Fig. 2. Adaptation to cold occurred in both age groups but to differing degrees. Young subjects showed a small but significant rise in serum corticosterone levels during the first half-hour of cold, but the levels then returned to basal values within the first hour. Aged subjects, in contrast, showed a significant elevation in serum corticosterone and circulating levels remained elevated throughout the 4 hours of cold exposure. That the declines in serum corticosterone reflect adaptation to the stress, rather than merely depletion of adrenal secretory capacity was demonstrated in subjecting the rats to a different stressor (2-minute ether exposure) at the end of the cold exposure and measuring the elevation in serum corticosterone. In both young and old rats, there were significant elevations in serum corticosterone (young: pre: 5.5 ± 1.3, post: 23.0 ± 3.1; old: pre: 12.5 ± 2.4, post: 26.7 ± 4.0).

Figure 3 shows the response to 1 hour of immobilization stress followed by the recovery from that stressor over the subsequent 90 minutes. As in section A of Fig. 1, there was an approximate 4-fold increase in corticosterone titer in both age groups during the 60 minutes of immobilization, and no significant differences were seen between the two groups during this period. However, a marked difference was seen during the period of recovery, in that in the young subjects, circulating levels of corticosterone declined to basal levels by an hour, whereas there were sustained elevations in serum corticosterone in aged subjects even at 90 minutes. In Fig. 4, Section A shows these data as a percentage decline during the recovery period, with 100% being equivalent to the corticosterone determination at the end of the stress period.

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The relative perseverance of circulating corticosterone in the aged subjects during the recovery period could not be attributed to any age-related change in corticosterone

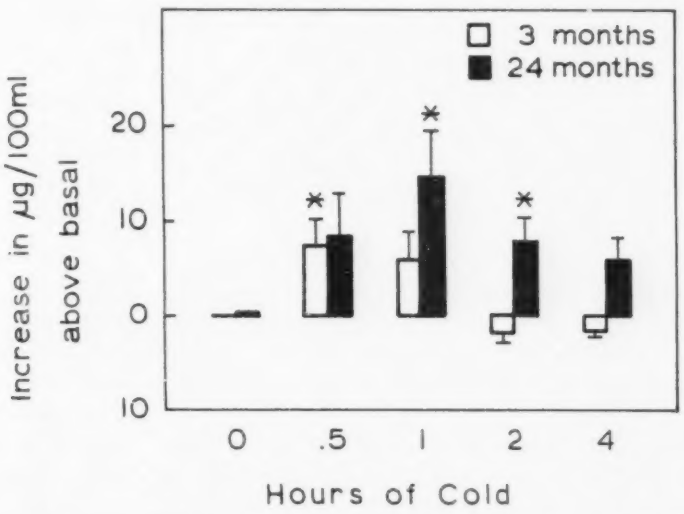

FIG. 2. Corticosterone titers in young and aged rats during four hours of cold exposure (4°C). Each bar indicates the change in titer from basal values at that time of day. An asterisk (*) indicates a significant elevation in cold-exposed subjects above basal values (95% confidence interval). n = 8, 7 for young and aged, respectively. Mean ± S.E.

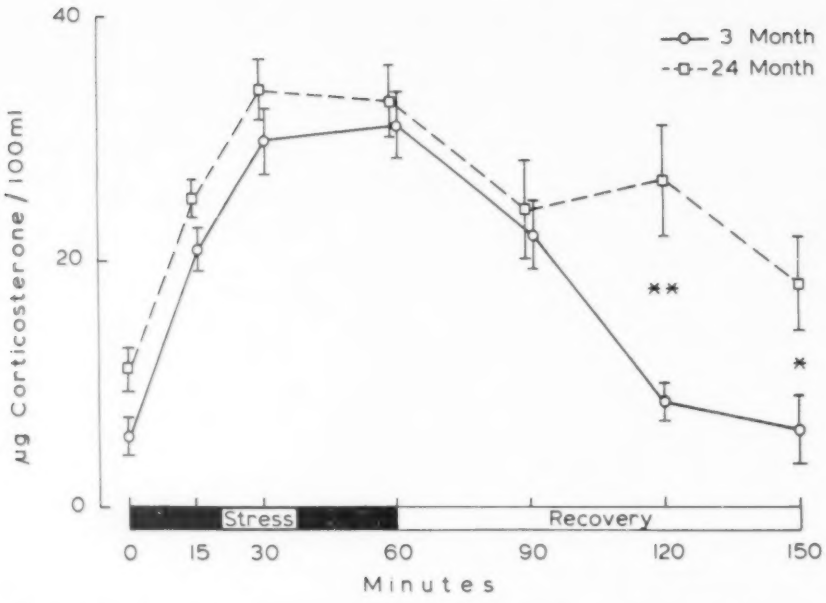

FIG. 3. Corticosterone titers in young and aged rats during 60 minutes of immobilization stress, followed by 90 post-stress minutes. Subjects were immobilized one hour into the light cycle. * and ** indicate significant differences between age-groups at the .05 and .01 level, respectively (two-tailed Student's t-test for independent samples). n = 22, 24 for young and aged, respectively. Mean ± S.E.

clearance rate. Half life of ^{3}H-corticosterone in the blood after injection was 15.6 ± 3.4 minutes for young and 15.2 ± 3.3 for old rats (n = 6). Such data suggest that the elevated levels during recovery in aged subjects are due to continued high rates of secretion and this is illustrated in Section B of Fig. 4 in which the data presented in Section A are corrected for clearance rate, leaving the amount of circulating corticosterone at those times attributable to secretion only (see equation 6 of discussion in Gurpide, 1972; w_{in} and w_{out} are assumed to contribute negligably to concentration).

DISCUSSION

A number of authors have characterized the adrenocortical axis of aging organisms as having diminished sensitivity to negative-feedback control by glucocorticoids (Riegle, 1976; Dilman, 1976). In the present studies, we have examined the status of the adrenocortical axis of the aged rat under a variety of states, in search of additional examples of an impairment in this feedback control. We have found an elevation of basal titers of corticosterone in aged subjects. In addition, we report that the capacity to appropriately terminate glucocorticoid secretion at the end of stress is diminished in aged animals.

Basal Function

The elevation of basal corticosterone concentration with age is a consistent finding in the literature (Hoff, *et al.*, 1981; Tang and Phillips, 1978; Wexler, 1976; Landfield *et al.*,

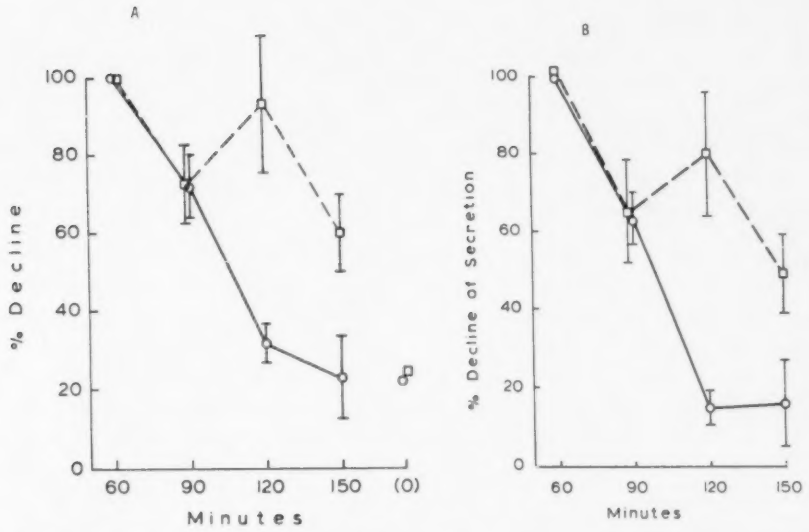

FIG. 4. Data from the recovery period of Fig. 3 expressed as percentage decline; 100% is equivalent to corticosterone titers at the end of the stress period. Section A: Percentage decline in serum titers of corticosterone. The circle and square at the (0) time-point represent the basal titers for each age-group as a percentage of corticosterone titers at the end of the stress period. Section B: Percentage decline in secretion of corticosterone. These data are derived from section A after correction for the clearance rate of corticosterone from the circulation. Mean ± S.E.

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1978 and 1979; Riegle, *et al.*, 1972 and 1973; Hess and Riegle, 1970 and 1972). It is apparent in both sexes, in a variety of strains and throughout the circadian cycle. In the present study, we find that the increase occurs progressively throughout the lifetime of the rat with an onset that appears during early adulthood (5 months). It should be noted, however, that given the magnitude of the increment and the magnitude of within-age variation, such a difference in the present data would not be statistically demonstrable were there only a comparison of youngest and oldest subjects. This appears to be the difficulty in several studies which conclude that there is no increase in basal corticosterone levels with age (Riegle, 1976; Tang and Phillips, 1978) and emphasizes the importance of monitoring more than two age groups. Parenthetically, it should also be noted that basal ACTH levels have also been reported to increase progressively from early adulthood through the rat's lifespan (Tang and Phillips, 1978).

Rats of the Fisher strain continue to grow throughout much of their lifetime and this results in an increase in blood volume with age. In view of this, the increase in circulating corticosterone observed in this study most likely represents a considerable increase in the adrenal secretion rate. Such a conclusion is reinforced by our observation that the clearance rate of corticosterone is similar in young and aged rats. The adrenal hypertrophies with age in the Fisher rat, but the percentage increase in adrenal weight is much less than that seen in body weight or blood volume (adrenal weight increase: 34%; body weight increase: 50%; blood volume increase; 63%). Thus, one may conclude that the increased basal titers of corticosterone during aging most likely represent a considerable increase in secretory rate per unit tissue of adrenal cortex. The physiologic significance of the elevated basal corticosterone titers is a matter of speculation. Chronic elevations of

glucocorticoids lead to impairment in immunocompetence (including thymic involution and lymphopenia), metabolic function (including hyperlipidemia, hypercholesteremia, hypertension and steroid diabetes) and neuronal plasticity (Munck, 1975; Wexler, 1976; Hoff, *et al.*, 1981). All of these pathophysiologic impairments are normal correlates of aging and thus may reflect a role played by the chronic elevation in glucocorticoid output.

Initiation of the stress-response

Aged subjects show no impairment in their capacity to respond to either cage transfer, cold, or immobilization stress. The time-course and magnitude of the stress-response are unchanged (Sections A and B of Fig. 1) and the stress-response of the aged subjects following chronic hypersecretion of corticosterone (Section C of Fig. 1) demonstrates that there is no age-related decline in corticosterone reserve capacity. A number of other studies have focused on this question, and, taken cumulatively, they also report no consistent age-related change in the speed or magnitude of the stress-response to a variety of stressors (Riegle and Hess, 1972 and 1973; Hess and Riegle, 1970 and 1972, Tang and Phillips, 1978). These data can be presented as percentage rise from basal levels of corticosterone and in some studies (although not the present) aged subjects show a decrease in percentage change following stress (Hess and Riegle, 1972; Tang and Phillips, 1978). However, this decreased percentage change is attributable solely to the initially elevated levels of the aged subjects; absolute magnitude of the stress-response is unimpaired. Thus, the physiologic significance of a decreased percentage change under these circumstances is difficult to ascertain (Andres and Tobin, 1977).

Riegle (1972) reported that aged subjects will show a larger response to ether stress following a period of chronic stress than will young subjects. He suggested that the elevations of corticosterone during chronic stress partially suppress secretion to subsequent stressors via negative feedback and that younger subjects were more sensitive to such negative feedback. While much evidence in both the present and other studies supports a general impairment of negative feedback control in the aged adrenocortical axis, the present data do not agree on this particular point concerning chronic stress effects. The possibility remains, however, that these differences may derive from the different stressors used in the two experiments.

It should be emphasized that all of these studies concern the aged male rat. The aged female rat does appear to manifest a corticosterone stress response that is substantially lower than that of the young female (Hess and Riegle, 1972; Wilson, 1981). There is a considerable sex difference in circulating corticosterone titers in the rat (Hess and Riegle, 1972), and it is of interest to note that young and old males, and old females, all have approximately equal stress responses, all being considerably smaller than that of young females (Riegle, 1973). All three of these groups also have low levels of circulating estrogens, relative to young females, and the possible involvement of estrogen in this system is a matter of current speculation.

Termination of the stress response

One new finding of this study is that aged subjects recover from and adapt to stress in a delayed and incomplete manner. This is seen in their failure following moderate cold exposure to turn off glucocorticoid secretion as rapidly as do young rats, or to terminate the secretion of corticosterone as rapidly at the termination of a stressor.

Rats have an initial corticosterone secretory response to moderate cold, but will ultimately adapt to the stressor and show titers that have returned to basal levels (Sakellaris and Vernikos-Danellis, 1975; Azmitia and McEwen, 1974). A similar degree of response and rate of adaptation was noted in the young rats in the present study. However, the aged subjects adapted much more slowly. The pattern of the aged subjects can be attributed to several mechanisms. First, aged rats subjectively perceive cold to be more stressful than do young rats and thus continue to secrete corticosterone for a longer period. Second, aged subjects perceived cold in the same manner as do young rats, but continue to secrete high levels of corticosterone because of a failure of negative feedback suppression of ACTH release by elevated serum levels of corticosterone once they have subjectively adapted. We favor the second hypothesis because of the supporting body of data listed above which suggests an impairment of negative feedback control in the aged adrenocortical axis, and because of the demonstration of a greater sensitivity to cold in young organisms than in aged, as measured by cognitive and sensorimotor function (Krag and Kountz, 1950; Fox, MacGibbon, Davies and Woodward, 1973; Watts, 1971; Horvath, Radcliffe, Hutt and Spurr, 1955).

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The impairment of negative feedback control is shown in the interpretably clearer data concerning recovery after the discrete termination of a stressor. Aged subjects show a delayed shut-off of corticosterone secretion when the stressor has abated. Under non-stressed condition, secretion of CRF and ACTH is influenced by circulating titers of corticosterone via negative feedback effects on brain and pituitary sites. During stress, the positive stimulatory signal of the stress on ACTH secretion will tend to override any inhibitory influences attributable to high circulating titers of corticosterone. After the end of a stressor, the decreased secretions of the adrenocortical axis are presumably due to the negative feedback effects of the elevated circulating corticosterone titers, this inhibitory signal no longer being overriden by a stress signal (Yates, Marsh and Maran, 1980; Dallman, 1979). An impairment of negative feedback control in the aged adrenocortical system would thus account for the delayed shut-off of secretion during the recovery and adaptation periods observed here.

In summary, we have reported three physiologic demonstrations of an impairment in the adrenocortical axis in the aging rat. The elevated basal corticosterone titers and the delayed adaptation to and recovery from stress can all be accounted for by a decreased sensitivity of brain-pituitary complex to the negative feedback actions of corticosterone. In a separate series of neurochemical studies, we have found that cell nuclear binding of corticosterone in the aged brain and pituitary is comparable to young brains in all regions, except for the amygdala, pituitary and hippocampus. These regions are frequently implicated in negative feedback control of ACTH release and sustain extensive depletion of neuronal binding sites during aging (Sapolsky *et al.*, 1983). The specificity and extent of this depletion leads us to hypothesize that such a depletion is a primary deficit of aging. With such a depletion of binding sites, these areas would have reduced levels of corticosterone receptor occupancy under a variety of conditions. Conceivably, this might lead to less inhibitory or more stimulatory influences of the neurons on CRF release, thereby producing the observed changes in basal, stressed and post-stress titers. We are currently investigating this hypothesis.

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THE EFFECT OF AGING ON THE DENSITY AND DISTRIBUTION OF ORAL MUCOSAL LANGERHANS CELLS

J.L. SCHWARTZ,* R. WEICHSELBAUM,* and S.R. FRIM**

*Department of Oral Medicine and Oral Pathology, Harvard School of Dental Medicine, Boston, MA.
**The Department of Radiation Therapy, Dana Farber Cancer Institute, Boston, MA.

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Abstract—Previous studies localizing the Langerhans cells in the hamster cheek pouch by ATPase staining have shown these cells to be distributed in a nonrandom pattern. The Langerhans cells have been found in both focal and interfocal arrangements. In this study we utilized two groups, Group 1 (six animals), was 5–6 weeks old, and Group 2 (six animals), was 78–80 weeks old. The Langerhans cells were stained for ATPase, following the epidermal stripping of the cheek pouch with 1% glacial acetic acid. The number of Langerhans was statistically decreased interfocally and there was a decrease in the number of focal aggregations, while there was a concomitant increase in the number of Langerhans cells in each unit. These alterations in the density and distribution of the Langerhans cells may suggest a lowering of the immune response, and increase susceptibility to irritants and carcinogenic agents in the oral cavity.

INTRODUCTION

LANGERHANS CELLS constitute a population of suprabasal elements in the epidermis and mucous membranes of mammals (Breathnach, Silvers, Smith and Heyner, 1967). These cells are regarded as macrophage equivalents in the skin and considered a critical cell for the presentation of exogenous antigens to T cells (Silberberg, Sinakin, Thorbecker, Baser, Rosenthal and Berezowsky, 1976). These cells also have functional characteristics of macrophages including Fc and C3 receptors, as well as Ia antigens and alloantigens of B cells (Stinge, Wolff-Schreimer, Pichler, Geschnait and Knapp, 1977), (Klareskog, Tjernlund, Forsum and Peterson, 1977), (Stinge, Katz, Abelson and Mann, 1978). Langerhans cells also play an integral role in delayed contact type of hypersensitivity reaction (Toews, Bergstresser and Streilein, 1980). Thus, the Langerhans cell is currently viewed as a sentinal cell which can participate effectively in the uptake, processing and presentations of antigen to the immune apparatus (Shelley and Juhlin, 1977). Schwartz, Solt, Pappo and Weichselbaum (1981) have recently described a characteristic distribution of populations of dendritic ATPase-rich Langerhans cells (LCs) which include focal aggregations of LCs with a diffuse interfocal distribution in whole mounts of hamster pouch epithelia (Schwartz *et al.*, 1981).

Requests for reprints and all correspondence should be addressed to: Dr. Joel Schwartz, Department of Oral Medicine and Oral Pathology, Harvard School of Dental Medicine, 188 Longwood Avenue, Boston, MA 02115.

Early studies on the antigenic processing by macrophage populations, "accessory cells," demonstrated that with aging there was a ten fold decrease in the ability of old mice to form antibody to injected sheep red blood cells (Makinodan and Adler, 1975). Associated with the decreased antigen processing was a failure of the capacity to localize antigen in antigenic processing sites, lymphoid follicles (Price and Makinodan, 1972).

In contrast, other studies utilizing peritoneal-derived macrophages from old mice, revealed an ability to engulf and digest optimally opsonized sheep red blood cells, an increased activity in cathepsin, B Glucuronidase, and acid phosphatase, the ability to initiate primary and secondary immune responses equal to young mice and the unaffected ability to activate T and B cells *in vitro* (Kay, 1976). Since Langerhans cells are abundant in the oral mucosa of the hamster buccal pouch, are macrophage equivalents, and may be important in the immune response, we studied the effects of the aging process on the distribution, number and morphology of Langerhans cells in whole mounts of oral mucosa from Syrian hamsters.

MATERIALS AND METHODS

Twelve non-inbred female golden Syrian hamsters (Mesocricetus auratus) served as experimental animals. The animals were obtained from Charles River Breeding Laboratories, Wilmington, MA. The experiments utilized two groups of hamsters. Group I and II consisted of six animals, each. Group I animals were 5–6 weeks of age while Group II animals were 78–80 weeks of age. The animals were housed five per plastic cage with wood chips for bedding. Environment was controlled with an alternating 12-hour light-dark cycle. Water and laboratory rat chow 4041 (Ralston Purina Co., St. Louis, Mo.) *ad libitum*.

Tissue Procurement and Epidermal Separation

After 5 weeks of treatment the animals were killed with carbon dioxide and the pouches were excised. The pouches were then immersed in cold 1% glacial acetic acid for one hour with constant stirring, and the epidermal specimens were separated from the underlying dermis using a wood throat stick.

ATPase Staining

ATPase staining of Langerhans cells was performed using a modification of the method used by Juhlin and Shelley (Juhlin and Shelley, 1977). The tissue was fixed in cacodylate formaldehyde solution for twenty minutes at 4°c, and then rinsed in ATP-Pb solution at 37°c for thirty minutes. After rinsing, it was immersed in ammonium sulfate for twenty minutes at room temperature. After rinsing again, it was mounted dermal side up in glycerin gelatin.

Langerhans Cell Density Determination

Each whole mount was traced on mm^2 graph paper for determination of whole mount area. Due to the large size of the whole mounts, the dimensions were given in cm^2. The average number of Langerhans cells per whole mount were calculated after determining Langerhans cell density in ten randomly selected high power fields (400X). The Langerhans cell densities were determined using a calibrated mm^2 grid. A maximum of 12.1 cm^2 and a minimum of 11 cm^2 with a total of 49.2 cm^2 from aged, and 70.7 cm^2 from young hamsters' epidermal cheek pouch tissue was analyzed. The difference in size of tissue was due to the difference in stripping of epidermis. The determination of number of focal aggregations reflect an approximation of the increased density of LCs in a localized region. Additionally, we have considered in our determination of the results the normal enlargement of the buccal pouch as the hamster ages.

Statistical Analysis

The mean and standard deviation of the area and number of Langerhans cells were ascertained for each group. Student's *t* test was also performed to determine statistical significance between each group of the number of Langerhans cells per cm^2 of area.

RESULTS

Microscopic examination of the ATPase whole mounts revealed a nonrandom distribution of Langerhans cells in a 5–6 week old, and 78–80 week old hamsters. Specifically, focal aggregates as well as a diffused array of Langerhans cells were seen in both groups of hamsters. Although the 78–80 week old hamster group exhibited a statistically significant decrease in the number of LCs located interfocally, and the number of focal units counted per cm² area (Table 1) (Figs. 1 and 2). The focal accumulations were not seen to be associated with any hair follicles or other skin appendages. It should be noted though that two varieties of focal aggregation were observed in the whole mount of both groups of normal hamsters. The first type of focal accumulations of LCs was found to be associated with vascularized epidermal outpouchings (Fig. 1), while the second form of aggregation observed was in the interfocal regions and appeared as a result of an elaboration of the normal interdigitating of dentritic processes of the Langerhans cells and was four or five times the densities of surrounding interfocally distributed Langerhans cells (Fig. 3). The number of these two types of aggregations varied in both groups but in general one tenth of the total number of focal aggregations observed was not associated with the epidermal evaginations of the whole mount. It was important to note, that the number of Langerhans cells in the focal units was statistically increased in the older, 78–80 week old hamsters (Fig. 4).

Morphologically, although there appears to be less fine dentritic processes which are interconnected, the processes themselves and the cell bodies of the Langerhans cells did not appear to be affected (Figs. 5 and 6).

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DISCUSSION

We have shown a significant decrease in the total number of Langerhans cells in the interfocal regions of epidermal whole mounts, as well as a statistically significant decrease

TABLE 1. DENSITY MEASUREMENTS OF LANGERHANS CELLS IN THE BUCCAL CHEEK POUCH OF NORMAL 5–6 WEEKS AND 78–80 WEEKS OLD HAMSTERS

Group	Animal	Total[a] Surface Area (cm²)	$\bar{x}$ Number of[b] Cells Interfocal/cm²	$\bar{x}$ Number of[c] Foci/cm²	$\bar{x}$ Number of[d] Cells in Foci/cm²
I (5–6 weeks)	6	70.7	672 ± 33.9	450.3 ± 47.4	567 ± 18.3
II (78–80 weeks)	6	49.2	223 ± 22.7	110.4 ± 23.9	1023.2 ± 97.7
p values (Student's *t* test)			$p \geq .001$	$p \geq .001$	$p \geq .001$

[a]Total surface area (cm²) was calculated following tracing of whole mounts on mm² graph paper.

[b]See text.

[c]The number of foci calculated included those aggregations of Langerhans cells observed associated with epidermal evagination and these the interfocal regions. LCs in these regions were two to three times the surrounding density of LCs and demonstrated increased interdigatation of dendritic process.

[d]This calculation is an approximation of the numbers of clearly discernible LCs in the focal unit. The LCs were determined to be an approximation because of areas in some foci that accumulated the ATP-Pb precipitate.

LCs = Langerhans Cells.

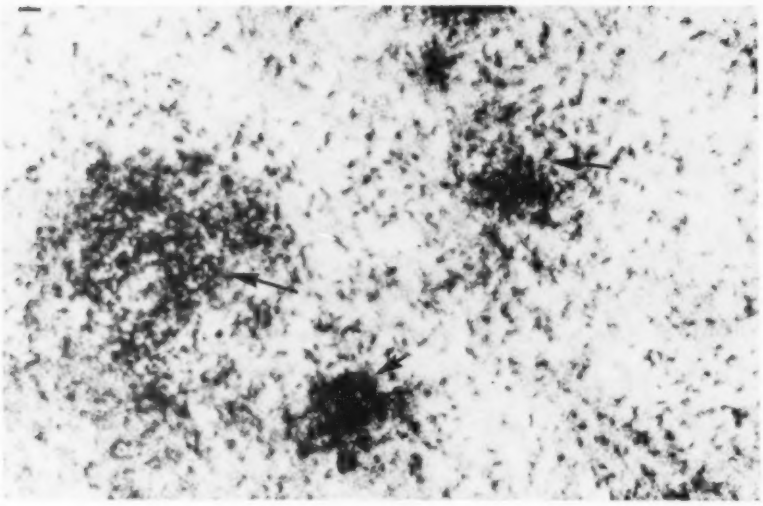

FIG. 1. Normal 5-6 week old epidermal cheek pouch tissue. The photomicrograph shows ATPase positive Langerhans cells distributed both in focal aggregates, and interfocally located. Note can also be made of the large quantities of precipitation product (ATP-Pb) found in the focal aggregate areas. Insert internal scale = .51 μM. (Arrows indicate focal areas).

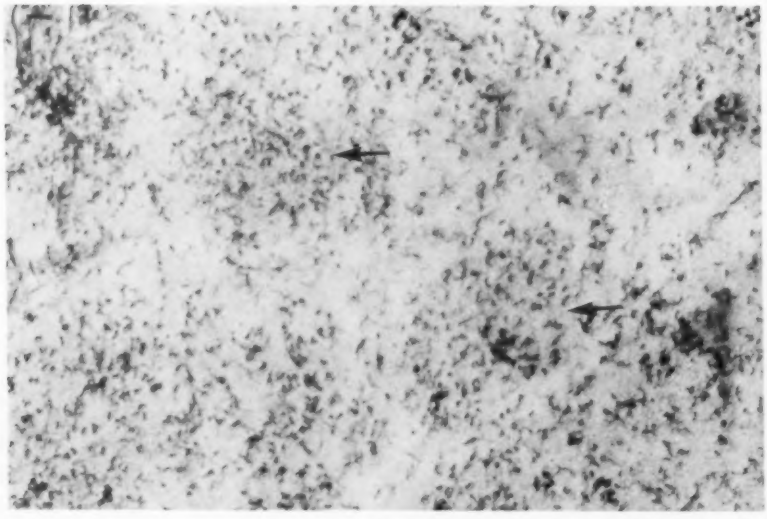

FIG. 2. Normal, 78-80 week old epidermal cheek pouch tissue. The photomicrograph shows ATPase positive Langerhans cells distributed in focal aggregates as well as interfocally located. In comparison to the younger epidermal whole mount sections, one observes less ATPase positive Langerhans cells interfocally, and a decrease in precipitation product (ATP-Pb). Insert internal scale = .45 μM. (Arrows indicate focal areas).

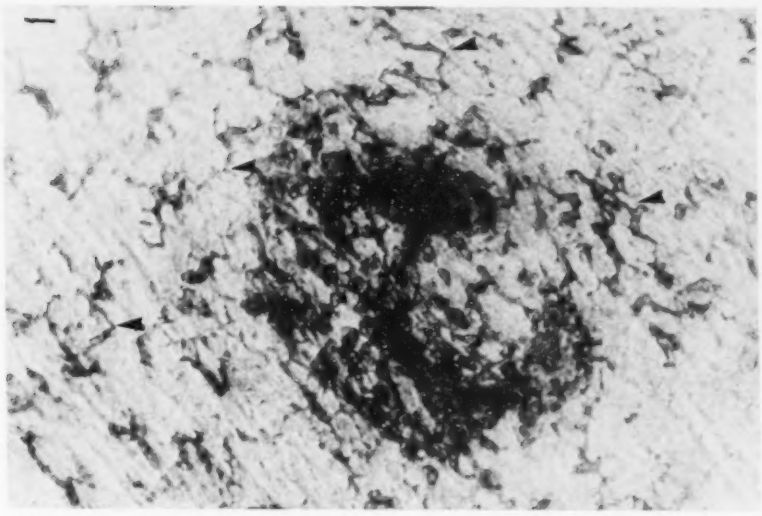

FIG. 3. A normal young focal aggregation of Langerhans cells exhibiting ATPase positivity. Note the interconnections of dendritic processes present. Insert internal scale = .71 μM. (Arrows indicates interconnections).

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in the number of focal aggregates of LCs in the older aged, 78–80 week hamster. In addition, we have noted a statistical increase of the number of Langerhans cells in the focal aggregations in the cheek pouch.

Langerhans cells have been implicated in critical immune functions (Bergstresser,

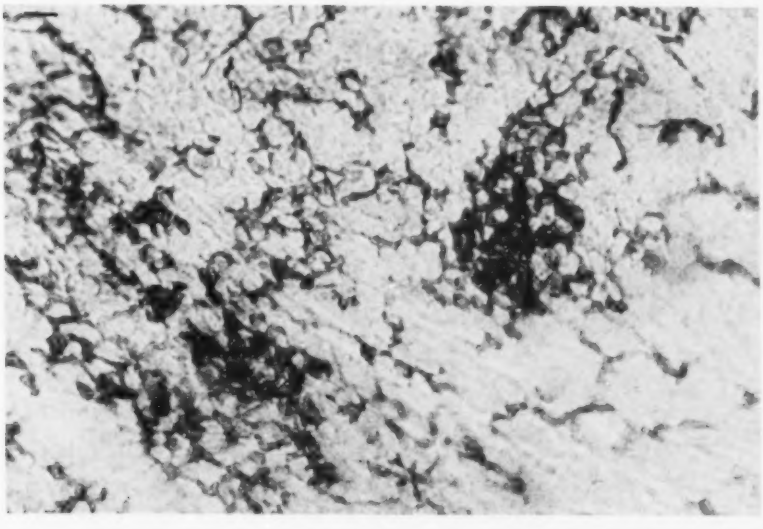

FIG. 4. Focal aggregation of Langerhans cells from a 78–80 week old epidermal whole mount. The photomicrograph shows a significant increase in Langerhans cells density. Insert internal scale = .71 μM.

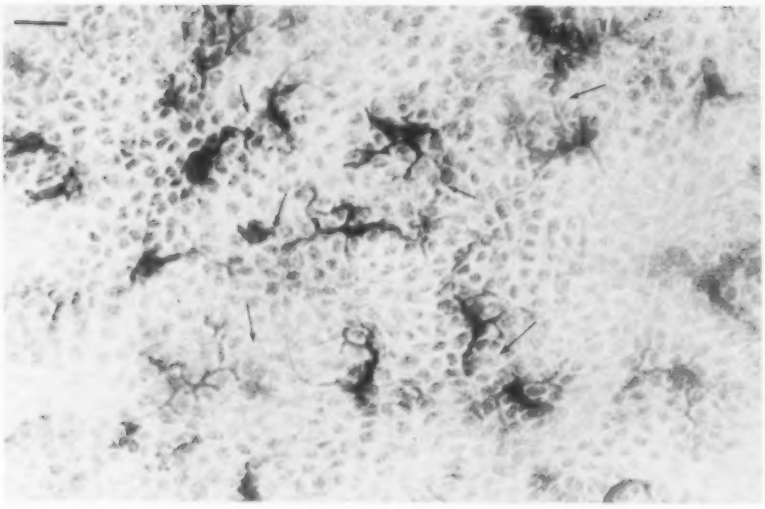

FIG. 5. In the older aged hamster epidermal whole mount, there is less interdigitating of the dendritic processes of the Langerhans cells located interfocally. Insert internal scale = .91 μM. (Areas indicated by arrows).

Fetcher and Streilrine, 1980) and were described by Bergstresser *et al.* in the cheek pouch. Thus, with the dramatic decrease in the number of Langerhans cells, which have been considered to be antigen processing elements of the immune system, there is the implication that there is a decrease in the ability of the older aged hamster to detect, especially weak

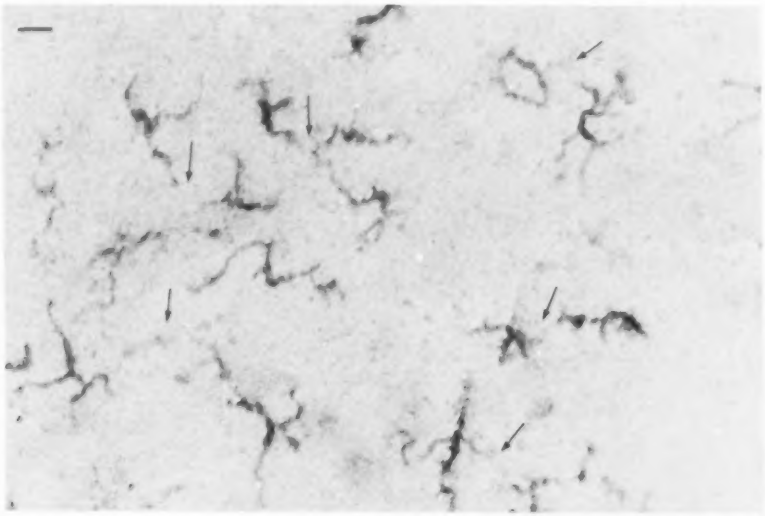

FIG. 6. In the cheek pouch tissue from the younger aged hamster, there is found interfocally a slight increase in the interconnections of dendritic processes of the Langerhans cells. Insert internal scale = .85 μM. (Areas indicated by arrows).

antigens, such as syngeneic tumor antigens. It is possible that the increase in the Langerhans cells in the focal units is an adaptive response to the loss of focal units and interfocal LCs to maintain local immunity.

It is interesting to note that it is predominantly the old aged patient who has had the oral mucosa irritated and effected by various exogenous agents from alcohol, tobacco smoke, and mechanical trauma from ill fitting dental applicances who develops oral carcinoma. Local immune suppressant may decrease the ability of the oral mucosa to eliminate cells which have already undergone malignant transformation. Therefore, studies on local immune function as a result of LCs number and distribution are important.

SUMMARY

In summary, we have shown that there was a nonrandom distribution of Langerhans cells in both the younger and older aged hamster buccal cheek pouch. The number of Langerhans cells is statistically decreased interfocally and there was a decrease in the number of focal aggregations, while there was a concomitant increase in the number of Langerhans cells in each focal unit. These alterations in density and distribution of the Langerhans cells may suggest a lowering of the local immune response, and increase susceptibility to irritants and carcinogenic agents in the oral cavity.

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DEVELOPMENTAL SWITCHES IN REITERATED GENES MAY REDUCE THE RATE OF AGE CHANGES IN DNA

ZHORES A. MEDVEDEV

Genetics Division, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

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Abstract—The possible role of the redundancy of genetic information in the regulation of the ageing rate has been discussed in several works. However, it was shown recently that the gene reiteration in most cases is represented by families of similar, but not identical genes. Their expression usually related to the different stages of development and when "early" embryonic or fetal genes are active, the "late" or adult genes are repressed. It is known that the DNA repair needs double stranded structure of DNA which is usual for inactive genes. Genes which are being transcribed and active are repressed by unwound, relaxed DNA which is less protected by the DNA repair enzymes. Aging of genetic information in somatic cells can be, therefore, considered as stage specific and alterations of "early" embryonic and fetal genes do not constitute the genetic load which influences the ageing rate of differentiated cells.

TEN YEARS AGO (Medvedev, 1972a,b), I proposed a hypothesis about the possible role of repeated nucleotide sequences in DNA in the evolution of the lifespan of differentiated cells. The idea of a possible correlation between gene reiteration and ageing rate was based on the simple assumption that the repetition of vital genes protects their functional information. I concluded that, all other conditions being equal, genomes with a relatively larger number of repetitious vital genes would be found in differentiated cells of longer-lived species. I also suggested that this correlation may not apply to regenerating and proliferating tissues, in which the lifespan of the individual cells is not a limiting factor. In this situation, the mutated or altered cells can be eliminated by selection.

The substantial increase of the knowledge about the nature of the gene reiteration phenomenon during the last decade and the finding that the reiterated genes are often represented by gene clusters which have different genes expressed at different stages of development, make it possible to modify now the hypothesis about possible relation between gene repetition and the ageing rate.

In addition to the protective role of simple gene repetition, the temporal separation of functionally similar genes expressed as embryonic, foetal and adult could have some ageing protective advantage as well. Such a temporal separation can more efficiently protect the latent adult genes from many different kinds of DNA changes and mutations during development, because the possibilities of DNA repair are lower for "working" genes which are being actively transcribed. Several attempts to find the correlation between the redun-

dancy of genetic information and the species-specific lifespan (Cutler, 1973), maturation time of plants (Bennet, 1972; Ivanov, 1978), or between the lifespan and the rate of age-related damage or loss of reiterated ribosomal genes (Strehler and Chang, 1979) were successful. However, the number of compared species was too small for animals, while for plants, where more than 700 species had been compared, the positive correlation was registered between the general size of the genome and the duration of reproductive maturation, not between the number of reiterated genes and the ageing rate.

Later, Ohno (Ohno, 1977; Ohno and Nagai, 1978) suggested that the redundancy of information, while protecting some genes, can actually make them the carriers of age-related changes in actively growing or proliferating tissues and tissue cultures. In such tissues the cellular selection can easily eliminate the deleterious mutations of single genes. Mutations which alter function sustained by a few copies out of many, are not likely to be deleterious, but may accumulate, resulting in more and more mistranslations and mistranscriptions, leading to a general deterioration typical of the ageing process.

Arguments about the possible role of gene reiteration in the control of the rate of ageing are further complicated by the more general discussion on the function and evolutionary origin of different types of gene repetition. The better studied cases of multiple copies of genes such as these for ribosomal and transfer RNAs and histones have been classified as "dosage repetitions" (Finnegan, Rubin, Young and Hogness, 1977; Long and Dawid, 1980). The level of gene reiteration in these cases is not related to the general lifespan, but to the type of early development (cleavage and blastula stages in sea urchin and amphibia), when very rapid cell division and growth requires very intensive synthesis of RNA and certain proteins, which could not be reached by few copies of these genes. Mammalian species which have comparatively slower rates of early development do not have such a high level of reiteration for these genes. Another type of gene repetition, called "variant repetition" (Dawid and Wahli, 1979), represents a more general case of a family of similar, but not identical genes. It was suggested that these variants, which are often clustered, evolved from a common ancestral gene by duplication and subsequent individual alterations (divergence) (Ohno and Nagi, 1978; Long and Dawid, 1980). Individual genes of the family may produce functionally related proteins, such as isozymes or heterogeneous groups of other proteins, or individual proteins, which serve different stages of development. They are represented by the "early" embryonic or "late" foetal, and the adult forms of certain proteins. The presence of stage-specific embryonic proteins at different developmental stages is probably common and has been reported many times (see reviews: Medvedev, 1970; Eppenberger, 1975; Neyfakh and Timofeeva, 1977). However, studies of the actual numbers of individual members of the gene clusters, were only started recently, and the number of known developmental gene families (now more than 10) is increasing every year (see reviews: Long and Dawid, 1980; Maniatis, Fritsch, Lauer and Lawn, 1980).

The necessity of transcriptional changes within one gene family during development is often functional. For example, in the case of globins, changes in expression of different globin genes are related to the different affinity to oxygen between foetal haemoglobins and the adult haemoglobin of the mother, or to other environmental differences in the fixing O_2 or CO_2. Changes in isozymes can be related to tissue specificity and these appear only during the later stages of development. However, the possible functional role of some known embryonic switches is not always as obvious as the haemoglobin case. Some specific embryonic forms of structural proteins, such as embryonic muscle proteins

(Villafranca, 1954), corneal stroma collagen (Malt and Bell, 1965) or keratin (Coleman, Hermann and Bess, 1965), do not actually function in embryos. Even when the functional role of gene reiteration of the family type can be explained, an additional advantage of this system of the temporal separation of functionally related products of transcription is also possible. I would like to indicate here one obvious advantage of transcriptional separation of embryonic, foetal and adult structural genes, even if their products are almost identical. Such a temporal separation prevents the mutation and many different kinds of DNA changes during development to play a serious role during adult life, because the DNA repair of latent genes is more efficient than those which are active and more vulnerable.

It is known that most DNA repair systems (excision repair, strand break rejoinings, postreplication repair and photoreactivation) need the double-stranded DNA. The intact DNA strand normally must be used as a template to restore breaks and other lesions in the complementary strand (Kornberg, 1980; Hanawalt, Cooper, Ganesan and Smith, 1979; Lehman and Karran, 1981). However, the typical double helix structure of DNA is possible only for silent untranscribed genes and the transcription in differentiated eukaryotic cells has quite a different picture than the transcription of prokaryotes, where a single RNA polymerase unwinds small parts of the DNA helix as it moves along. Some models of the structure of active chromatin of specialized cells (Crick, 1971; Palter, Foe and Alberts, 1979) postulate that either transcribed portions or at least the recognition sites where transcription starts are represented by unpaired single-stranded stretches of double-stranded DNA. Crick (1971) called this suggestion "the unpairing postulate." There are now many data which show that the actively transcribed genes have different state of DNA than untranscribed silent genes, specially in terminally differentiated cells (Weintraub and Groudine, 1976; Bonner, Wallace, Sargen, Murphy and Dube, 1978; Weisbrod, Groudine and Weintraub, 1980; Kolato, 1981). In this case, the transcriptionally active regions of DNA have a "relaxed" unwound structure and can be cut by nucleases that specifically react with single-stranded DNA and destroy the active genes. In terminally differentiated cells, this single-stranded state of actively transcribed genes is a normal, not a transitionary state.

The molecular mechanism of unwinding of DNA and the possible role of high mobility non-histone proteins 1 and 2 in the destabilizing and unwinding of the DNA helix and the HMG proteins 14 and 17 in the interaction with single DNA strand of actively transcribed chromatin are now under intensive study (Javeherian, Sadeghi and Liu, 1979; Isackson, Fishback, Bidney and Reek, 1979; Bakaev, Schmatchenko and Georgiev, 1979; Weisbrod, Groudine and Weintraub, 1980). At the same time, the single-stranded sections of DNA, if damaged, cannot be repaired in the same way as double-stranded DNA. Except the very rare cases of somatic recombination, the repair of single-stranded DNA is impossible. One may, therefore, conclude that the active transcribed genes are much more vulnerable for different lesions than the inactive double-stranded sections of the genome. The correlation between the activity of species-specific excision repair of DNA and maximal lifespan (Hart and Setlow, 1974; Hart, D'Ambrosino, Ng and Modak, 1979a and Hart, Sacher and Hoskins, 1979b; Francis, Lee and Regan, 1981; Treton and Courtois, 1982), demonstrates the importance of double-stranded DNA structure to express the evolutionary potential of the DNA repair capacity. The increased ability to repair DNA cannot, therefore, influence the lifespan by preserving the integrity of active (constitutive) eukaryotic genes. The importance of more efficient repair of DNA damage of un-

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transcribed genes for the lifespan potential can be seen only for genes which are not transcribed at one stage of development, but will be transcribed later. It is quite possible that the correlation between the excision repair activity and the lifespan reflects the correlation between the duration of pre-reproductive development and the total lifespan, which has been discussed in gerontology for many years. The longer the duration of the development (embryonic and post-embryonic) stage, the more efficient the preservation of integrity of latent genes needs to be, since the genes may be expressed only after several months or even years of development.

The higher repair potential is also important for the protection of inducible genes which are transcribed only after induction by certain factors. It was shown (Resnick and Holliday, 1971) that when the UV-irradiation exposure damages the DNA of *Ustilago maydis* just before the induction of the synthesis of inducible nitrate reductase, the synthesis of the enzyme is delayed, and significant part of the newly synthesized enzyme is defective. The authors consider the pyrimidine dimers as responsible for delay of transcription, while other UV-induced changes leading to molecular alteration of the enzyme.

The advantage of temporal expression of reiterated vital genes can also be seen when mosaicism of tissues or organs arises due to DNA damage (or mutations) at early stages of development. Mutation at the blastomere stage will affect 50% of all somatic cells; mutation at the stage of 8 cell embryo will affect 12.5% of somatic cells and mutation of a single cell at the blastula stage can still affect the whole tissue or organ. If a mutation in an embryonic cell is not lethal, it still could lead to functional or morphological abnormalities in later life in the case where the same genes are active during embryonic and post-embryonic development. If embryonic genes are switched off and "adult" genes in the same gene family are switched on, the load of somatic mutations of early development do not seriously influence the normal functions of post-natal life.

Earlier (Medvedev, 1972a,b) I suggested that, all other conditions being equal, genomes with higher frequency for the reiteration of vital genes, must have higher "life expectancy" in differentiated cells. Now one can add that these families of reiterated genes, which have individual genes transcribed (and exposed to the more damage-prone single-stranded state) at different periods of development would be able to support one or other biochemical function longer than gene clusters which do not separate the expression of individual genes in time.

The greater the vulnerability to damage of genes which are being transcribed (due to difficulties in repair of the single-stranded DNA), together with the large "somatic progeny" of embryonic cells, make clear the evolutionary advantage of a special developmental type of gene reiteration where functionally replaceable genes are transcribed at different stages of development. The integrity of genes which are dormant during embryonic development is maintained by DNA repair systems and protected from single-strand alterations. However, where genes are protected by simple reiteration, such protective reiteration would be important for the most active stages of development. The "dosage repetition" of key genes would provide a very rapid and more perfect passage through the most sensitive early developmental stages. Even a low level of reiteration of other embryonic genes would raise the changes of completing the developmental program to a state where "adult genes," already less redundant, would be expressed.

It has been shown that the synthesis of messenger RNAs during the bluastula stage of development is mostly from higher reiterated DNA sequences (Volvson and Vorobjev, 1972). The proportion of reiterated genes in mRNA synthesis drops 20% by the late

gastrula stage (Gelderman, Rake and Britten, 1971). But the method of DNA/mRNA hybridization which had been used in these studies cannot distinguish between really single genes and genes with low reiteration rates. The suggestion that the protective role of temporal gene reiteration in development may also explain the existence of long spacer regions between functionally similar or identical genes. It is possible that, in addition to some regulation of switches, the longer spacers provide means to achieve temporal transcription of coding sections of DNA. This hypothesis also provides an explanation why the very high activity of DNA repair enzymes in embryonic cells sharply decline after birth (Gensler, 1981) and why the DNA repair enzymes have a low activity in long-lived terminally differentiated cells, like nervous or muscle cells (Gensler and Bernstein, 1981; Lehman and Karran, 1981). Active genes in such cells cannot be repaired and the double-stranded sections of the genome do not have functional importance. The possibility of repair of inducible genes which pass from active (single DNA strand) form to inactive, double-stranded structure periodically, remains important in differentiated cells as well.

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LIVER POLYPLOIDY:

Influence of Age and of Dietary Restriction

HILDEGARD E. ENESCO and JOHN SAMBORSKY

Department of Biology, Sir George Williams Campus, Concordia University,
1455 de Maisonneuve Boulevard West, Montreal, Quebec H3G 1M8, Canada

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Abstract—Polyploidy increases with age in mammalian liver. Since dietary restriction increases lifespan, the question posed in this study was whether liver polyploidy would be influenced by dietary protein restriction. Restricted mice were fed a 4% protein diet, while control mice received a 26% protein diet. Polyploidy was determined from measurements of nuclear diameter on liver of 3 week, 1½, 3, 10, 20 and 24½ month old Swiss albino mice. The percentage of polyploid cells increased steadily with age. The rate of increase, expressed as the polyploidization index, was greater in the fully fed control animals as compared to the animals on protein restriction. Protein restriction thus retards the age associated process of polyploidization of liver nuclei. Nuclear diameter increased slightly with age, but was not influenced by protein restriction.

INTRODUCTION

THE PURPOSE of this study was to examine changes in the level of polyploidy in mouse liver during aging, and to determine whether polyploidy would be influenced by dietary protein restriction. It is well established that dietary restriction extends the lifespan of mice and rats, whether it is carried out by restricting food intake (McCay, Sperling and Barnes, 1943; Ross, 1959) or by restricting protein in the diet (Leto, Kokkonen and Barrows, 1976). Polyploidy in the liver is known to increase with age (Alfert and Geshwind, 1958; Shima and Sugaraha, 1976a) and is one of the best documented examples of an age-related change at the cellular level.

This study was designed to determine whether or not dietary restriction would alter the rate of polyploid cell formation in mouse liver, and extends an earlier study (Enesco and Samborsky, 1981). Animals on dietary restriction have been reported to have a younger appearance than control animals (McCay, *et al.*, 1943), to have a slower rate of aging of collagen (Everitt, 1971), and to have a lower rate of accumulation of lipofuscin (Enesco and Kruk, 1981). If dietary restriction slows the rate of aging, we might predict that it would also slow the rate of accumulation of polyploid cells in the liver.

Correspondence and requests for reprints should be addressed to Dr. H. Enesco.

MATERIALS AND METHODS

Animals and diets

Male Swiss albino mice were obtained from Canadian Breeding Farms, St. Constant, Quebec at 3 weeks of age. They were housed three per cage in an animal room maintained at 72°F on a regular 12 hour light: 12 hours dark cycle. They were maintained on Purina chow for 1 week, until they had attained an average body weight of 15 g. At 1 month of age, control mice were placed on Teklad diet TD78071 containing 26% protein, while the other group intended for restriction was placed on Teklad diet TD78072, containing 4% protein. These diets are produced by Teklad Co., Madison, Wisconsin. The protein source in these test diets is casein, and the diets are available to the mice *ad libitum*. The construction of the diets has been described by Leto, *et al.* (1976). Water was available *ad libitum*.

Histological preparation

Groups of 2 to 5 mice were used for each age and each diet. One group of mice was sacrificed at 3 weeks of age, prior to being placed on the special diets. The mice on the special diets were sacrificed at 1½, 3, 10, 20 and 24½ months. Mice were sacrificed by cervical dislocation, the liver was dissected out, and 1 mm^3 pieces were fixed in Bouin's solution for 24–48 hours. Liver samples were passed through a graded alcohol series, cleared in xylol and embedded in paraffin. The tissues were sectioned at 10 um, and stained with hematoxylin and eosin.

Histometrics

A calibrated ocular micrometer, placed in the eye piece of a Zeiss Ultraphot microscope, was used to measure the diameter of randomly selected hepatocytes at 100x magnification. In most instances there were 5 animals per group, and measurements were made on a minimum of 25 nuclei per animal. Since there were only two animals per group for the oldest mice, a minimum of 100 nuclei per animal was measured. Nuclear diameter measurements were used to calculate nuclear volume. These measurements were then evaluated to see whether the nuclei formed the regular groups which characterize a polyploid series according to the criteria of Helwig-Larsen, (1952).

Methods of analysis

For statistical analysis contingency tables, chi-square, and unpaired t-tests were used as described by Sokal and Rohlf (1969). The polyploidization index was calculated using the method of Shima and Sugahara (1976a).

RESULTS

Since measurements of nuclear diameter were made on randomly selected liver parenchymal nuclei (hepatocytes) our first task was to determine whether the nuclei could be grouped into a regular polyploid series, using the criteria of Helweg-Larsen (1952). In Fig. 1, actual nuclear diameter measurements have been arranged as a histogram to show the size distribution of nuclei by 0.5 μM increments. The data shown are from 10-month-old mice on the 26% protein diet, which had a wide range of nuclear sizes. The shape of the histogram shows that there are distinct size classes. The curved lines superimposed on the histogram represent the theoretical or expected distribution of these size classes generated from the mean and standard deviation of the observed diameter classes. This presentation emphasizes the regularity of the size increments. To verify that the classes of nuclei we observed represent a 1:2:4:8 sequence we ploted nuclear volume against ploidy class and found that the correlation coefficient for the regression was positive and greater than 1. The correlation between our data and the expected sequence was significant at $p < 0.05$. Thus, we have clear evidence of a polyploid series based on size distribution of hepatocyte nuclei.

Table 1 is presented as a complete summary of all nuclear diameter measurements grouped according to age, diet and ploidy class. With this data in hand, we proceeded to

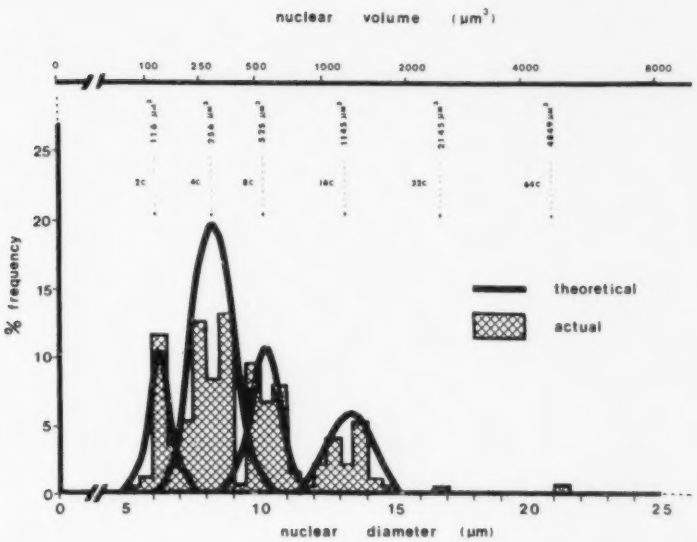

FIG. 1. The frequency distribution of actual and theoretical class of nuclei make a regular progression characteristic of a polyploid series.

ask the following questions: (1) Is nuclear diameter influenced by age or by diet? (2) Is ploidy distribution influenced by age or by diet?

In examining the data, one has the impression that nuclear size increases with age. To test whether a trend existed, we grouped the 3 week and 6 week nuclear diameter

TABLE 1. NUCLEAR DIAMETER MEASUREMENTS GROUPED ACCORDING TO AGE, DIET AND PLOIDY CLASS

Diet	Age	Average nuclear diameter in μM					
		2C	4C	8C	16C	32C	64C
pre-diet	3 wks	5.90	7.67	9.71	–	–	–
4% protein	1½ mo	5.63	7.31	9.77	–	–	–
	3 mo	6.09	7.97	9.66	12.38	–	–
	10 mo	5.87	7.54	9.79	12.93	–	–
	20 mo	6.23	7.83	9.71	12.25	15.17	–
	24½ mo	6.32	7.85	9.87	12.00	14.50	–
	Average	6.03	7.70	9.76	12.39	14.84	–
26% protein	1½ mo	5.82	7.54	9.33	12.83	–	–
	3 mo	6.14	7.85	9.89	12.60	15.10	–
	10 mo	6.05	7.87	10.01	12.98	16.00	21.00
	20 mo	6.25	8.01	9.83	12.45	15.50	–
	24½ mo	6.40	7.91	9.92	12.49	15.02	–
	Average	6.13	7.84	9.79	12.67	15.41	21.00

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TABLE 2. THE AVERAGE NUCLEAR DIAMETER FOR MICE ON BOTH 4% AND 26% DIET INCREASES SLIGHTLY WITH AGE. (THE "YOUNG" GROUP IS THE 3 AND 6 WEEK OLD MICE, WHILE THE "OLD" GROUP IS THE 3, 10, 20 AND 24.5 MONTH OLD GROUPS.)

	Average diameter in μM		
Diet and age	2C	4C	8C
4% young	5.77	7.49	9.74
4% old	6.13*	7.80	9.76
26% young	5.86	7.61	9.52
26% old	6.21*	7.91*	9.91*

*Significantly different from young at $p < .05$.

measurements together to form a "young" group, and the 3, 10, 20 and 24½ month measurements together to form an "old" group. The measurements from animals on the 4% and 26% protein diets were evaluated separately. The data from the 3 week prediet animals were included in both "young" groups. Table 2 shows our analysis of the average nuclear diameter measurements for the "young" and the "old" groups. For animals on the low protein diet, an unpaired t-test showed that only the diploid nuclei showed a significant ($p < 0.02$) size increase with age, while the 4C and 8C nuclei did not show any significant change in diameter with age. In contrast, there was a sigificant increase ($p < 0.05$) in size with age in 2C, 4C and 8C nuclei in the animals on the 26% protein diet.

To determine whether nuclear diameter was influenced by diet, data from mice of all ages for each ploidy group were averaged. Nuclear diameter was then compared between the two dietary groups for each ploidy class by constructing a contingency table and calculating the Chi-square value. This analysis showed that there was no significant relation between diet and nuclear diameter.

We will now turn to a consideration of ploidy distribution as influenced by age and by diet. Table 3 shows the percentage of nuclei in each ploidy class at each age for mice on

TABLE 3. PERCENTAGE OF NUCLEI IN EACH PLOIDY CLASS

Diet	Age	Percentage of Nuclei					
		2C	4C	8C	16C	32C	64C
pre-diet	3 wk	77.7	19.4	2.9	–	–	–
4% protein	6 wk	77.3	17.9	4.8	–	–	–
	3 mo	51.4	28.2	18.1	2.3	–	–
	10 mo	57.3	32.8	4.6	5.3	–	–
	20 mo	34.6	33.0	28.5	2.2	1.7	–
	24.5 mo	30.1	48.9	20.4	0.2	0.2	–
26% protein	6 wk	52.3	43.6	2.9	1.2	–	–
	3 mo	56.3	29.0	12.5	1.1	0.6	–
	10 mo	16.8	39.5	26.3	15.3	1.6	0.5
	20 mo	18.9	34.0	36.4	9.8	1.0	–
	24.5 mo	5.8	27.6	56.0	9.4	1.2	–

the 4% protein diet and on the 26% protein diet. It is evident from Table 3 that polyploidy increases with age, and that the frequency of diploid nuclei declines with age, regardless of diet.

Figure 2 emphasizes graphically the steady decline in the percentage of diploid nuclei with age, and allows comparison of the rate of decline in diploid nuclei, which is more rapid for mice on the 26% protein diet as compared to mice on the 4% protein diet.

Figure 3 is a histogram in which the frequency of all classes of nuclei is presented for mice on both diets. Visual inspection of the histograms emphasizes the decline in frequency of diploid nuclei and the increasing frequency of polyploid nuclei of all classes with age for mice on both the 4% protein and on the 26% protein diet. Statistical analysis was carried out by constructing a contingency table and calculating Chi square values. This analysis confirmed that there was a very significant ($p < 0.05$) relation between age and ploidy distribution for mice both on the 4% protein diet and on the 26% protein diet.

The last, and perhaps the most important question that we asked was whether ploidy distribution was subject to influence by diet. A visual comparison of the histogram in Fig. 3 suggests that higher ploidy classes appear earlier in mice on the 26% protein diet than for mice on the 4% protein diet. Statistical analysis confirms that there is a significant ($p < 0.05$) effect of diet on ploidy distribution. Again, a contingency table was con-

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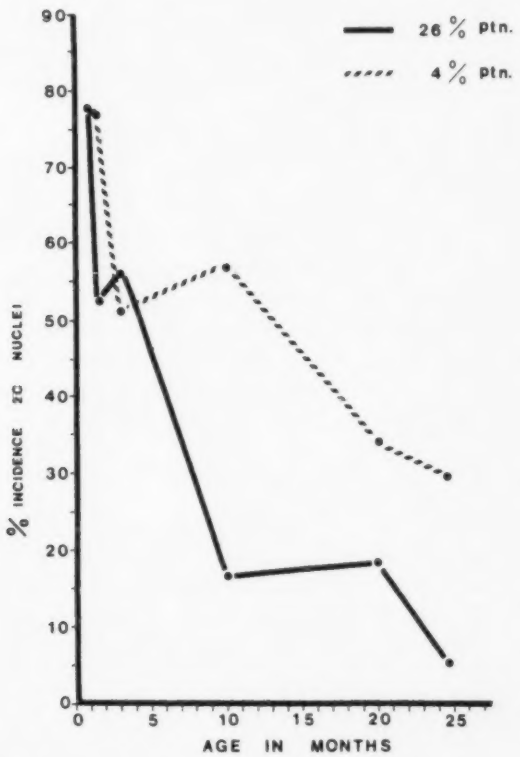

FIG. 2. The percentage of diploid nuclei steadily declines with age in both control and restricted mice.

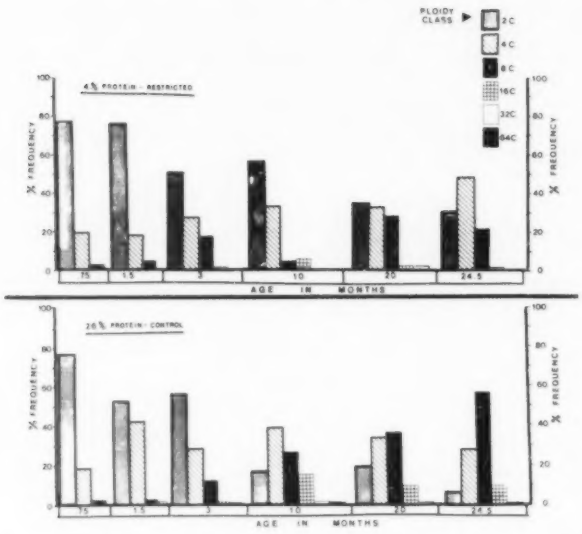

FIG. 3. The frequency distribution of nuclei of each ploidy class for mice on each diet at each age is presented.

structed and Chi-square values were obtained on data at all points in the lifespan to perform this analysis. When this same analysis was carried out at each individual age, significant ($p < 0.05$) differences in ploidy distribution as a function of diet were confirmed for mice at 1½, 10, 20, and 24½ months of age. Only in the 3 month group was the distribution of nuclear classes similar for mice on both diets.

To evaluate the progression of polyploidy throughout the lifespan as a continuum, we turned to the method of Shima and Sugahara (1976a) for calculation of the polyploidization index, which can then be expressed graphically as the slope of an exponential function. Figure 4 shows the data expressed in this manner. It is clear that the slope of the polyploidization index is much different for animals on the two different diets. The slope of the curve for our control mice on the 26% protein diet is very similar to the data published by Shima and Sugahara (1976a). This presentation of our data confirms that polyploidization takes place at a slower rate in mice on protein restriction.

DISCUSSION

Our finding that nuclear diameter increases significantly with age in hepatocytes of fully fed control mice is consistent with other reports in the literature. Studies by Carriere and Patterson (1962) and by Nadal and Zajdela (1966a) both report that the size of diploid liver nuclei is smaller in neonatal and in very young rats than in adult rats. In the present study we found a statistically significant age-related size increase in nuclear diameter of mouse hepatocytes for diploid, tetraploid and octaploid nuclei in fully fed control animals. This same trend toward an age-related size increase was found for nuclei from restricted mice, although the difference was statistically significant only for diploid nuclei. Protein is the major constituent of the nucleus, and makes up 82.9% of the dry mass of liver nuclei (Allfrey, Daly and Mirsky, 1955). Since there is no significant difference in the

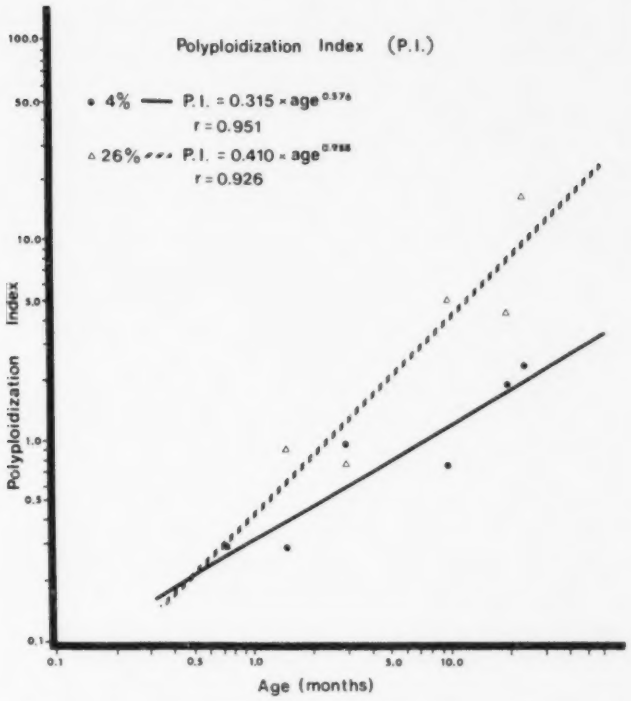

FIG. 4. The slope of the exponential function of the polyploidization index is compared for mice on the control (26%) and on the restricted (4%) diet.

size of the nuclei in protein restricted as compared to fully fed control mice, the data suggests that the protein restriction must not result in lower levels of the essential nuclear proteins.

Several previous studies have been carried out to determine the effect of short term (3 to 6 weeks) food restriction on polyploidization of young rats. These studies all produced different results. Bass and Dunn (1957) placed growing rats on a restricted diet for 21 days, and found that there was no significant difference in the amount of liver polyploidy between control and restricted animals at the end of the 21 day period. Nadal and Zajdella (1966b) placed 4 week old rats on restricted diets for 5–6 weeks at a restriction level sufficient to stop growth. They report that the liver of the restricted rats failed to acquire any polyploid nuclei. When adult rats were placed on protein deficient diets for 14–60 days, there was no change in the ploidy distribution in the liver. Naora (1957) placed young growing rats on growth restricting diet for a 33 day period. At the end of this period, the control animals had 90.6% 4N nuclei, while the restricted rats had only 26.7% 4N nuclei.

Because these previous studies all involved short-term food deprivation, and because of the differing results, it is difficult to relate our results to these earlier reports. Nevertheless, two of these studies point to the conclusion that dietary restriction either retards the rate of polyploidization or stops its occurrence. Our study differs from these earlier studies in that dietary restriction was imposed throughout the study period; up to 24½

months. Polyploidy level was consistently lower throughout the lifespan of the restricted animals in the present study. The use of nuclear diameter measurements to evaluate ploidy, as was done in the present study, have been validated by the work of Helwig-Larsen (1952), Epstein (1967) and Epstein and Gatens (1967).

In trying to understand the meaning of polyploidization, investigators have asked the following questions: Is polyploidization related to growth? Is it related to a change in cell function? Is it a response to an increased physiological or metabolic load? How is it related to aging? We will examine these considerations in relation to the data presented here.

With respect to growth, we can ask whether the reduced ploidy level could be related to the smaller size of the restricted animals. Even though growth ceases earlier in the restricted animals than it does in the control animals, a gradual increase in polyploidization occurs throughout the lifespan of both control and restricted animals. For this reason, polyploidization does not appear to be correlated with the growth rate or ultimate size reached by the animals.

Since polyploidy reduces the surface to volume ratio of cells (Epstein, 1967), various investigators have considered the question of whether or not an increase in polyploidy reduces cell function. Harris (1971) has shown that growth rates are identical for 2N as compared to 4N, 8N and 16N kidney cells. Collins (1978) reports that the rate of RNA synthesis is nearly equivalent for hepatocyte of all ploidy classes. The somatic mutation theory of aging (Gordon, 1974) would lead us to expect that polyploid cells should have a longer lifespan than diploid ones, because they would have less chance of accumulating recessive mutations. However, Thompson and Holliday (1978) report that diploid and polyploid human fibroblast have the same or similar lifespans. In summary, there is no evidence that polyploidy produces a decline in cell function.

On the other hand, there is considerable evidence which suggests that polyploidy may be a response to an increased physiological load. Polyploidization is increased after exposure to irradiation (Shima and Sugahara, 1976a), in tumor-bearing animals (Naora and Naora 1967), during repeated liver regeneration (Carriere 1964), and in other instances of compensatory hyperplasia or hypertrophy (Paulini and Sonntag, 1977). Polyploidy is increased in rat kidney following unilateral nephrectomy (Paulini, *et al.*, 1970). Polyploid nuclei increase in percentage with hypertrophy of the human heart (Sandritter and Scomazzoni, 1964). Polyploidy increases during enhanced stimulation of endocrine glands (Gilbert and Pfitzer, 1977). The frequency of polyploid cells is greater in the beta-cells of diabetic mice as compared to control mice (Pohl and Swartz, 1979). Although hypophysectomy will retard polyploidization, Geshwind, Alfert and Schooley (1958) report that liver regeneration in hypophysectomized rats is accompanied by an increase in polyploidy. They reason from this observation that no pituitary hormone is indispensible for the development of polyploidy. Paulini and Mohr (1975) suggest that "the appearance of polyploid cell nuclei is the result of an increased functional load." All the examples cited would fit with this conclusion. Physiological theories of aging point to declines in functional status with age and to decreases in homeostatic ability (Shock, 1974; Comfort 1979), which would bring out the need to respond to an increased physiological load. The numerous studies which relate increased polyploidization of liver to increased age have been reviewed by Carriere (1969) and by Brodsky and Uryvaeva (1977). Our results are in agreement with those of Shima and Sugahara (1976a) that polyploidization of mouse hepatocyte nuclei is an age-dependent phenomenon that continues progressively

throughout the lifespan of the animal. The slower rate of age-related polyploidization that we observed in the restricted mice may be a reflection of the altered rate of aging. Gahan (1977) has proposed that a move to higher ploidy levels is a strategy to reduce deleterious changes with age. Our results suggest that these changes are taking place at a slower rate in the protein restricted mice. We should qualify our conclusions about diet and lifespan with the realization that the time when mice are placed on the restricted diet and the degree of restriction are critical in lifespan studies.

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LONG-TERM OBSERVATIONS ON THE EFFECT OF POLYADENYLIC ACID IN MICE OF DIFFERENT AGES

LÁSZLÓ PÉNZES, EDIT BEREGI and OTTÓ REGIUS

Gerontology Center, Semmelweis Medical School, Budapest, Hungary

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Abstract—In order to better life performance, polyadenylic acid (poly (A)) was given intraperitoneally to CBA/Ca mice for almost a two-year period. This substance, as one of the components of double-stranded polynucleotides (like poly A:U), is known to improve some immune responses of the aging organism. Five approaches (changes in body-weight, adaptation to cold stress, biological half-life of body proteins, mortality and pathology) were applied to test the effects of this substance on life performance. It was found that the beneficial effects of double-stranded polynucleotides cannot be mimicked by polyadenylic acid only, despite its anti-senescence effect, namely, it accelerates the apparent protein turnover, cf., biological half-life. Polyadenylic acid shortens life-expectancy (because of the higher mortality rate of mice). Possible mechanisms of these actions are discussed.

INTRODUCTION

NOWADAYS A very promising possibility is at hand, that the rate of aging can be mitigated to some extent through immunoengineering with advancing age. It seems as an effective approach to give adjuvants, as induction agents, which have potential immune enhancing activity in the aged organism (Boyse and Abbott, 1975; Braun, Yajima and Ishizuka, 1970; Jaroslow and Taliaferro, 1956; Johnson, 1973; Kent, 1977; Makinodan, 1979; Scheid, Hofmann, Komuro, Hammerling, Abbott, Boyse, Cohen, Hooper, Schuloff and Goldstein, 1973). The synthetic double-stranded polynucleotides, like poly A:U, poly C:G and poly I:C proved to be efficient in restoring some immune functions in old mice.

We must point out that the polyadenylic acid, poly (A), is one of the components of the double-stranded polynucleotides concerned and has a unique role in nuclear metabolism (Darnell, Jellinek and Molloy, 1973; Puckett, Chambers and Darnell, 1975; Bergmann and Brawerman, 1977). Some of these mean that blocking of poly (A) synthesis stops the appearance of labeled mRNA in the cytoplasm (Darnell, *et al.* 1973). In other words, polyadenylation and poly (A) chain extension may stabilize the mRNA by protecting it from degradation (Rose, Jacob and Kumar, 1979). The role of aging in the nuclear RNA manufacturing was studied by Yannarell, Schumm and Webb (1977) who concluded that a decreased poly (A) content of the putative mRNA might be relevant to the problems of cellular aging.

Address all correspondence and requests for reprints to Dr. László Pénzes.

In the light of the foregoing, the aim of the present paper is to call attention to how the polyadenylic acid influences some parameters, namely, body-weight, cold tolerance, apparent turnover, mortality and pathology of mice during aging.

MATERIALS AND METHODS

Our studies are based on the barrier-maintained inbred mice strain CBA/Calati, obtained from the Laboratory Animal Institute, (LATI) Gödöllő, Hungary. The SPF animals (whose pathology was monitored regularly) were male and monthly injected ip. with a 150/μg dose of polyadenylic acid (REANAL, Budapest) dissolved in physiological saline, pro animal. A total of 147 animals (between 28 and 33 g) were used and the mice were fed a standard laboratory chow (LATI) and supplied with tap water *ad lib*. Body-weights were regularly recorded in every month. Five approaches (changes in body weight, cold tolerance, ^{75}Se-selenomethionine biological half-life, mortality and pathology) were applied to test the effects of poly(A) on life performance. In order to test the mice for their adaptive capacity against acute cold stress, the rectal temperatures of the animals were measured three times in the periods concerned; i.e., before, just after the cold (-4–-2°C) and one hour later, respectively. ^{75}Se-selenomethionine turnover was used as an index to measure protein metabolism. This was done and evaluated according to Yousef and Johnson (1970 a,b); Yousef and Luick (1969). Using ^{75}Se-selenomethionine as a tracer, we must point out that its reutilization is comparatively high. Thus the present indication "apparent" (biological half-life) seemed to be essential.

For histologial examinations, small parts of the liver, spleen and kidney were fixed in 96% ethanol, embedded in paraffin. The 4 μ sections were stained with haematoxylin-eosin, Congo-red, gentiane-violette and PAS. For electronmicroscopical investigations, small pieces of the spleen were freshly fixed in cold glutaraldehyde, then in osmium tetroxide, finally embedded in Araldite. Ultrathin sections were prepared by LKB-Ultratrome III, contrasted with lead citrate and examined in a JEM 100 C electron microscope. The electronmicroscopical examinations were used for verification of the light microscopical observations. Data were evaluated by using the Student "t" test and the level of significance was chosen as $p < 0.05$.

RESULTS

Figure 1 shows the body weight changes of the untreated (control) and poly(A)-treated mice during the experiment. (The animals were treated with poly(A) from the 6th month

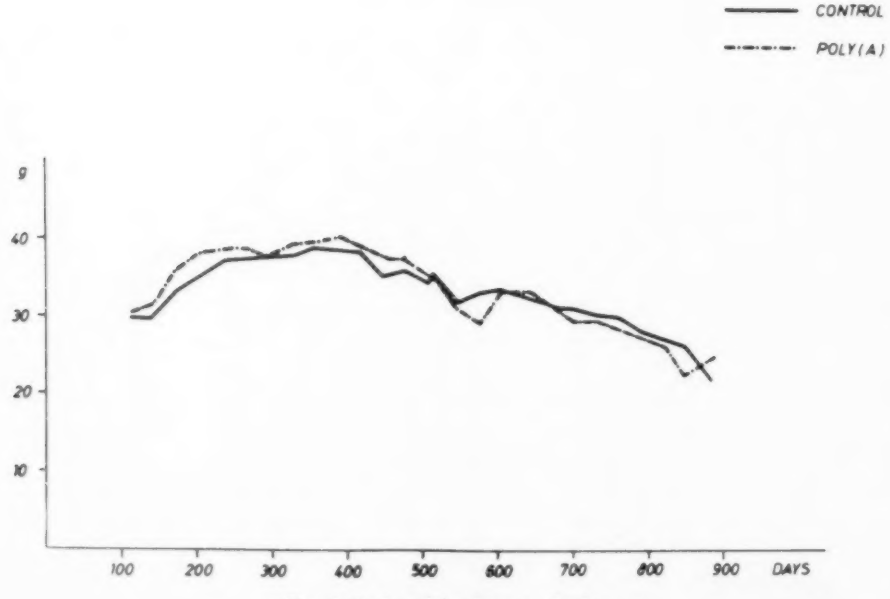

FIG. 1. Body weight changes of mice.

onwards.) The data indicate that there were no considerable differences among the body weights of the control and experimental groups.

Figure 2 demonstrates the changes in body (rectal), temperatures of the control and poly(A)-treated animals. One might observe that there is a decreasing trend of rectal temperature as time progresses (first columns of each age-group). As the figure shows, the experimental group reveals to some extent different temperature values, after the 1 hour cold exposure than that of the controls. (These temperature values are shown by the second and third column of each age-group. Comparatively similar standard deviations were measured in the poly(A) group as well; however, these are not drawn for the sake of better comprehension.) All the controls decreased (during cold) and increased (after cold) their rectal temperature significantly ($0.01 > p$). The response to the cold of the poly(A) treated animals were found more markedly in the last two age-groups ($0.05 > p$).

Figure 3 discloses the biokinetical data of the ^{75}Se-selenomethionine metabolism of the two groups. The data clearly demonstrate that the $T_{½}$ (biological half-life) values increase in all animals as time advances, but not at the same rate. In the case of the controls the half-life values elevated nearly linearly with increasing age, however, the augmentation of the $T_{½}$ values is lessened in the poly(A) treated mice. The difference between the controls and experimental group becomes more marked as time advances thus the variance proved to be significant ($0.05 > p$) at 23.2 month of the experiment. No significant changes were recorded at 27.8 month, possibly due to the relatively small number of animals.

The mortality curves are demonstrated in Fig. 4. The highest mortality was observed in the poly(A) group when the animals reached their age of roughly 1½ years. The percentile survivors of the controls were apparently more, namely by 10%.

These findings can partly be explained by the main pathological observations which are

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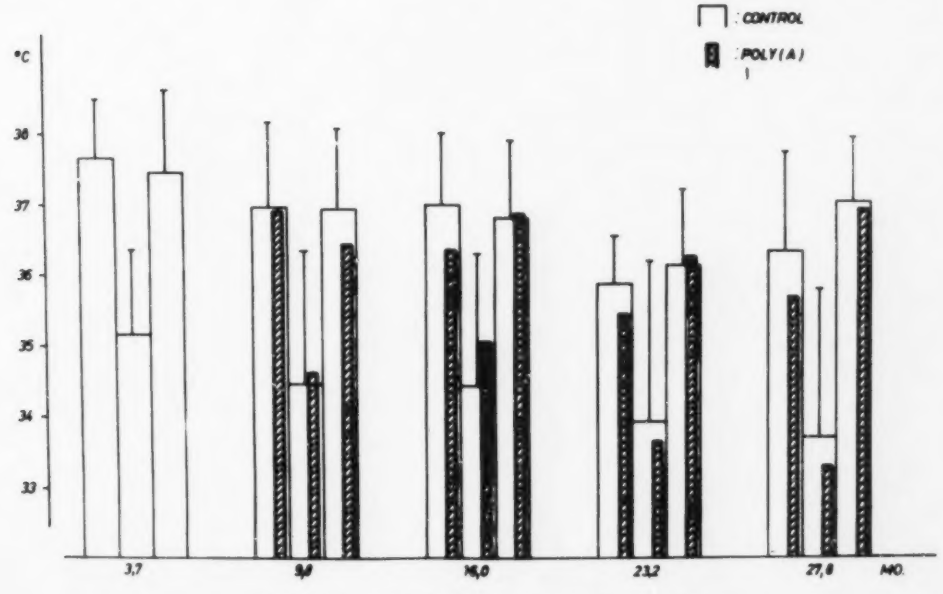

FIG. 2. Changes in body temperature (Mean ± SD).

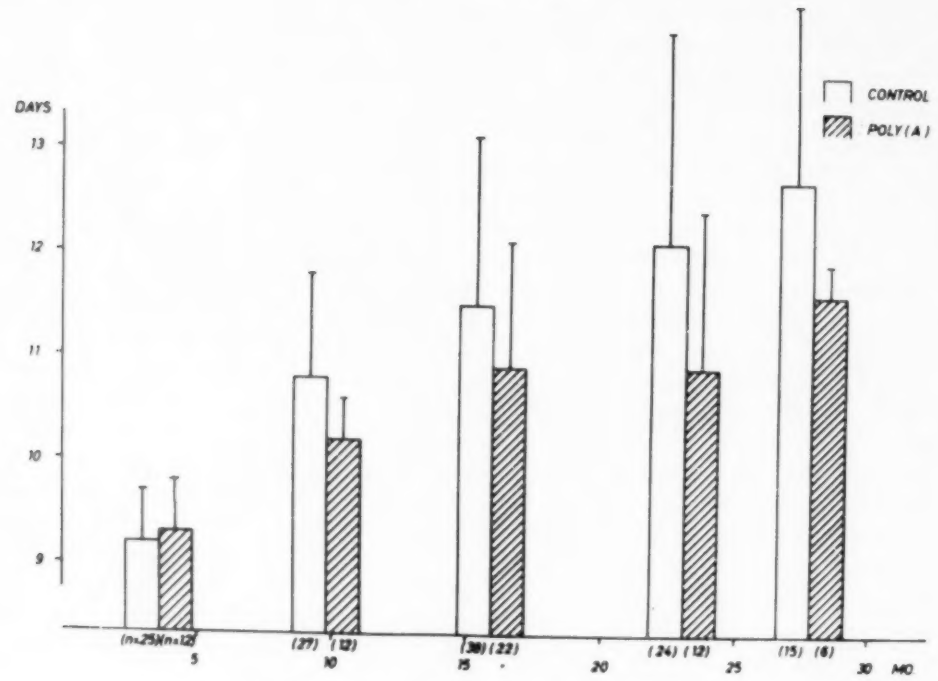

FIG. 3. Apparent biological half life (^{75}Se-selenomethionine $T_{½}$).

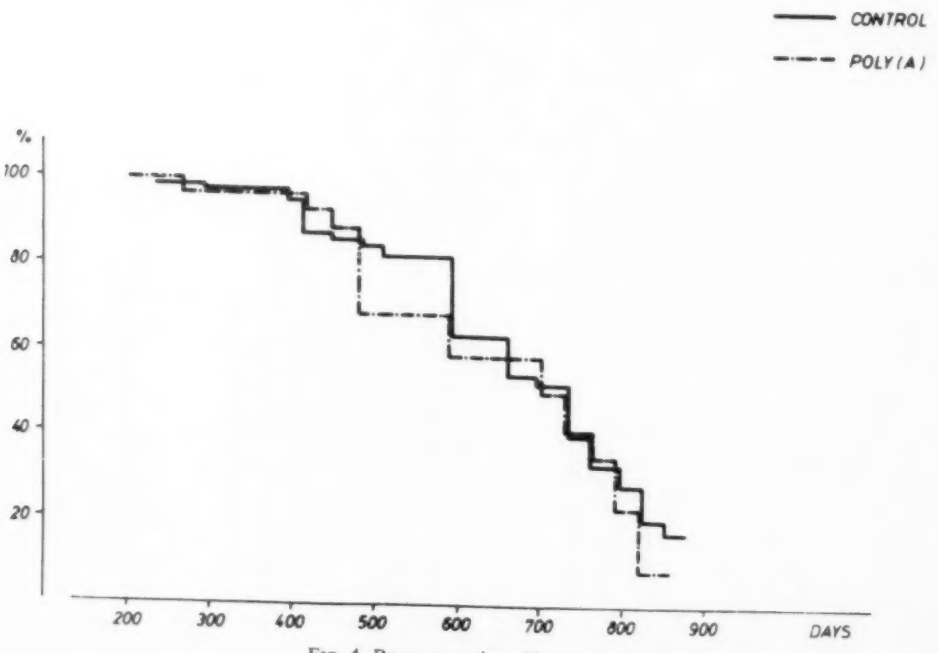

FIG. 4. Percent survivorship vs. age.

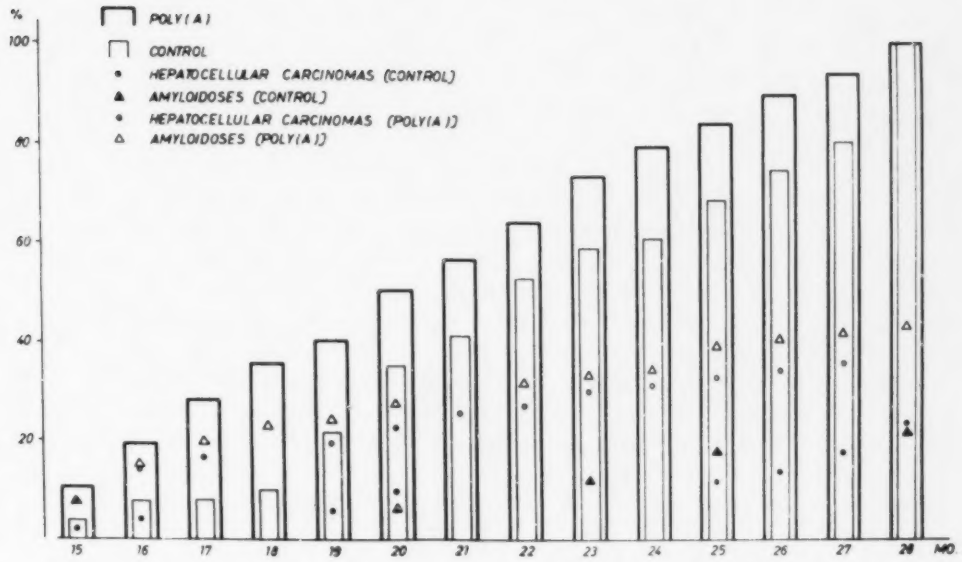

FIG. 5. Age-dependent pathological alterations.

shown in Fig. 5. The poly(A) group exhibited a significantly greater occurrence of age-dependent pathological alterations of which the proportion of hepatocellular carcinomas and amyloidoses proved to be considerably higher than that of the controls. On the other hand, these changes occurred much earlier than in the untreated, control mice.

DISCUSSION

The results show that the polyadenylic acid exhibited a controversial effect on the parameters investigated in the present study. In respect to the cold tolerance of the animals, all mice kept the rectal temperature in a similar way beyond the age of 93 weeks. In old age, there seems even a mild hypercompensation after the cold exposure. It should be added, however, that the poly(A) treated mice tolerated the cold stress more efficiently. According to the relatively low number of the experimental animals, the poly(A) treated mice were roughly half so many as that of the controls, the differences between the rectal temperatures were not so explicitly significant $0.02 > p$) than that of the untreated animals ($0.01 > p$). On the whole, the control mechanisms change as the animals age.

Furthermore, the data show that the biological half-life of ^{75}Se-selenomethionine increases (approx. by 40%) in the control ranging from 3.7 to 27.8 months of age. This is in accordance with the findings of Sobel and Bowman (1971) who established that following injection with ^{14}C-lysine the $T_{½}$ value increased more than 30% in male mice ranging in age from 320 to 750 days of age.

One of the main conclusions of this work is that the apparent protein turnover decreases most dramatically in young and young-adult mice up to 480 days of age and then remains relatively less changed beyond the ages of 16 months. This finding fairly harmonizes with the observations of Yousef and Johnson 1970(a) who established that protein metabolism, as measured by the ^{75}Se-selenomethionine turnover rate method, shows a

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decreasing trend during lifespan of the rat. We have to add to these, that the greater apparent protein turnover corresponds to the rapid body weight gain of mice, roughly in the first one-third of the lifespan concerned. Comparatively less elevation in apparent biological half-life ($T_{½}$ in days) in poly(A) treated mice was observed during the course of the 830-day experimental period.

Poly(A) administration alone does not confirm the findings to better life performance as that of the beneficial effects of double-stranded polynucleotides. Despite its anti-senescence influence, in the sense of accelerating the apparent protein turnover, poly(A) shortens lifespan because of the higher mortality rates; i.e., increased aging is a by-product of increased metabolism. The present data thus confirm the fact that the metabolic factor can influence the onset and progress of age-dependent diseases (amyloidoses and hepatocellular carcinomas).

This metabolic change could partly be attributable to the altered condition of the thyroid. It is known that in senescence there is a decrease in thyroid function. According to Eleftheriou (1975) the constant decline in rectal temperature of inbred mice directly correlates to thyroid activity. The higher apparent protein turnover in the present case indicates that some intrinsic factors within the gland or a higher responsiveness to the levels of TSH even to TRH cannot be ruled out.

From the present observations it seems very probable that the characteristics already mentioned as well as the immune restoring actions previously described of one of the substances in question depend firstly on the double-stranded polynucleotide molecule as a whole and not on one component only, like polyadenylic acid.

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AGE-RELATED CHANGES IN THE HYPOTHALAMIC-PITUITARY-TESTICULAR FUNCTION OF THE RAT

E. BEDRAK,[1] Z. CHAP[2] and R. BROWN

Department of Biology, Ben-Gurion University of the Negev, Beer-Sheva 84120, Israel

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Abstract—The activity of the hypothalamic-pituitary-testicular axis was investigated in 3–4 months (young) and 24 months (old) rats. The results clearly demonstrate that aging reduces ($p < 0.01$) the hypothalamic content of gonadotrophin releasing hormone (GnRH), decreases the capacity of the pituitary ($p < 0.01$) to synthesize and or release follicle stimulating hormone and luteinizing hormone (LH) following a single stimulation of GnRH (50 ng/100 g body weight), lowers the capacity of the testes to produce testosterone ($p < 0.01$) following multiple subcutaneous injections of human chorionic gonadotrophin (hCG 3IU/100 g body weight for 3 consecutive days), decreases the number of Leydig cell LH receptors and decreases the *in vitro* responsiveness of the hCG-challenged Leydig cell to synthesize testosterone ($p < 0.01$). These phenomena are independent of a major alteration in the capacity of the hCG challenged Leydig cell to produce adenosine 3′, 5′-monophosphate. It is concluded that the decline in testicular activity accompanying senescence is not inherent to the testes only but is also associated with alteration in the function of the hypothalamus and pituitary which eventually lead to the loss of fecundity.

INTRODUCTION

RECENTLY IT has been demonstrated in man that advancing age goes hand in hand with reduced sexual activity which is not accompanied by decreased serum testosterone (T) concentration. However, it is accompanied by a lower pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH) response to a challenge of gonadotrophin releasing hormone (GnRH) as well as a decreased sensitivity of the Leydig cell to human chorionic gonadotrophin (hCG) stimulation (Harman, Martin and Tsitouras, 1979). The fact that T levels were not decreased with aging is surprising and contradictory to previously published data (Rubens, Dhont and Vermeulen, 1974). On the other hand, experiments with the male rat support the thesis that the decline in the testicular function which accompanies aging is characterized by a significant decline in serum T (Ghanadian, Lewis and Chrisholm, 1975; Riegle and Meites, 1976; Chan, Leathem and Esashi, 1977; Miller and Riegle, 1978; Gray, 1978; Bethea and Walker, 1979; Kaler and Neaven, 1981;

[1]To whom correspondence and request of reprints should be addressed.

[2]In partial fulfilment of requirements of the degree of the Ph.D. Current address: Diabetes Research, St. Luke's Episcopal Hospital, Houston, Texas 77025.

Chan, Mcfarlin and Zummo, 1981), decreased sexual arouse (Gray, Smith, Dorsa and Davidson, 1981) and lowered thyroidal activity (Klug and Adelman, 1979). The reduction in the testicular function and lowered fecundity in the senescent male rat is associated, in part, with a significant decline in activity of some of the steroidogenic enzymes in the testicular tissue (Leathem and Albrecht, 1974; Chan *et al.*, 1977; Bedrak, Chap and Fried, 1979) as well as a reduced response to hCG injection, *in vivo* (Chan *et al.*, 1977; Miller and Riegle, 1978; Kaler and Neaves, 1981). There appears to be some doubt, however, whether the reduced testicular function of the aging rat stems primarily from an intrinsic Leydig cell defect (Tsitouras, Kowatch and Harman, 1979; Chan *et al.*, 1981) without any aberration in the function of the hypothalamic-pituitary moieties which controls the testes' activity. The present study was undertaken to assess the integrity of the hypothalamic-pituitary-testicular axis (axis) with respect to the control of T production in the aging male. The results indicate that the reduced gonadal function accompanying aging is not inherent to the testes only, but also is associated with an alteration in the function of the other moieties of the axis.

MATERIALS AND METHODS

Animals

Sprague-Dawley derived male rats from the departmental colony were housed in air-conditioned quarters illuminated between 06:00 and 20:00 h. Tap water and rat chow were available *ad libitum* throughout the experiment. All animals were weighed weekly as an indicative criterion for growth and development. Generally, comparative experiments were conducted between rats of two age groups: 3–4 month-old (young) and 24 month-old rats (old). When rats of other age groups were used their age was specified in the relevant experiment.

Blood and tissue collections

All animals were bled between 09:00 and 12:00 h by cardiac puncture under chloral hydrate anesthesia (0.3 ml of 10% chloral hydrate per 100 g body weight) and killed for collection of tissues. The blood was allowed to clot for 2 h at room temperature and placed in a refrigerator at 4°C for 24 h. Serum was collected by centrifugation at 800 g for 10 min at 2–4°C and stored at −20°C until hormone assays. Immediately after killing the testes were placed in an ice-cold medium containing Hanks salts (Bio-Lab, Jerusalem) and 0.1% bovine serum albumin (BSA). The Leydig cells were isolated shortly thereafter and prepared for the various incubation experiments. The hypothalami, consisting primarily of the medial basal hypothalamus and the arcuate nucleus, were removed according to the coordinates of de Groot (1972) and treated according to the procedure of Koch and Baram (1976).

Isolation of the Leydig cells

Siliconized glassware was used for the isolation of the Leydig cells and for experimentation with them. The isolation of cells was done in the cold (2–4°C) except when indicated otherwise. After careful decapsulation, each pair of testes was transferred into a 50 ml erlenmeyer containing 10 ml medium 199 fortified with 0.5 mg/ml collagenase (Clostridiopeptidase Type 1A, Sigma Chemical Company, Saint Louis, Missouri, U.S.A.) and 1% BSA, pH 7.5. The tissue was then incubated at 37°C with gentle shaking (30 strokes/min) until the seminiferous tubules were moderately loosened from the decapsulated testes. Washed cells were separated from the tubules according to the method of Dufau, Mendelson and Catt, (1974) and Van Damme, Robertson and Diczfalusy, (1974) with the following modifications: following the loosening of the seminiferous tubules the contents of the erlenmeyer were transferred to a 15 ml round-bottom tube. The seminiferous tubules were allowed to settle down to the bottom of the tube (approximately 1–2 min) and the upper aqueous phase (containing the Leydig cells) was transferred, with a pasteur pipet, to a 30 ml round-bottom tube which was kept in an ice bath. Five ml of medium 199 were added to the tubes containing the seminiferous tubules, the tubes were covered with parafilim and gently inverted 10 times. The seminiferous tubules were allowed to settle down to the bottom of the tube (1–2 min) and the upper phase was transferred to the 30 ml round-bottom tube containing the original harvest of the Leydig cells. This step of the isolation procedure was repeated twice. The combined harvest of the Leydig cells was centrifuged at 500 g at 2–4°C, for 3 min and the upper phase was discarded. The precipitate, containing the Leydig cells, was washed twice with 10 ml of L-15 medium (Bio-Lab, Jerusalem) containing 2% fetal calf serum (FCS). These washes facilitated the removal of the undesired proteolytic enzymes from the

Leydig cells' preparation (Moyle and Rammachandran, 1973). The washed cells were centrifuged and resuspended in 1–3 ml of the appropriate incubation-medium (depending upon the experiment to which they were intended, which will be described later). The total number of cells in each preparation was determined by counting; in Neubauer hemocytometers; the percent of Leydig cells by differential counting after 3β-hydroxysteroid dehydrogenase staining and the percent of live cells by trypan blue exclusion as described by Dufau and Catt (1978). From the above measurements, the number of living Leydig cells in each preparation was calculated.

Production of T by the isolated Leydig cell

The experiments for the determination of T secretion were done in triplicate and in a total volume of 1.0 ml medium 199, pH 7.45; fortified with 1% BSA, 1% FCS, 20 mM HEPES buffer (equimolar portion of Hepes, Bes and Tes, Sigma Chemical Company), 0.1 mM 1-methyl-3-isobutyl-1-methyl xanthine (IBMX); containing the freshly prepared Leydig cells (1-3 × 10^5 living Leydig cells) and an appropriate concentration of hCG. The incubation was carried out under an atmosphere of 95% O_2 and 5% CO_2 for 3 h with shaking (90 strokes/min) at a temperature of 34°C. The reaction was stopped by the transfer of the tubes to an ice bath and the immediate extraction of the media with 2 × 5 ml double distilled diethyl ether. The extract was evaporated to dryness and T was determined by the radioimmunoassay (RIA) previously described (Bedrak *et al.*, 1979).

Adenosine 3',5'-monophosphate (c-AMP) production by the Leydig cell

Cell suspensions (1-3 × 10^5 living Leydig cells) were incubated, under an atmosphere of 95% O_2 and 5% CO_2, with shaking (60 strokes/min) at 37°C for 20 min in Krebs-Ringer-bicarbonate buffer (0.4 ml, pH 7.4) containing 1 mg/ml glucose, 0.1% BSA, 0.1 mM IBMX and an appropriate level of hCG. The reaction was stopped by the addition of 0.4 ml of 0.05 M sodium acetate buffer, pH 4.0 and immediate boiling for 5 min. Samples were frozen and kept at −20°C until the assay. Cyclic AMP content was assayed by the method of Gilman (1970) with one modification. Namely, the separation between the free and bound nucleotide was done by the use of dextran-coated-charcoal as used in the RIA for T (Bedrak *et al.*, 1979). This modification simplified the procedure without effecting the sensitivity and accuracy of the assay.

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Assay for hCG receptors in either the Leydig cell or testicular protein

Iodination of hCG (13,450 unit/mg, Center for Population Research, NIH, Batch CR-121) was carried out by the lactoperoxidase method described by Miyachi, Vaitukaitis, Nieschlag and Lipsett, (1972). The biological activity of the labeled preparation was determined by assay in adenylate cyclase system (Salomon, Yanovsky, Mintz, Amir and Lindner, 1977) and used to determine the mass of active hormone. Based on this mass, the specific radioactivity of labeled hCG was found to be 20–50 μCi/μg.

Binding of ^{125}I-hCG to Leydig cells was assayed as described by Nimrod, Erickson and Ryan, (1977). Briefly, cell suspensions (100 μl containing 1-3 × 10^5 living Leydig cells) were incubated for 90 min at 34.5°C in a shaking incubator (45 strokes/min) together with 100 μl of buffer containing 2.5 ng/ml ^{125}I-hCG and varying amounts of unlabeled hCG. Non-specific binding was determined by incubating cells in the presence of a 100 fold excess of unlabeled hCG and this value was subtracted from all values of bound radioactivity prior to the calculation of binding parameters (Scatchard, 1949; Nimrod, *et al.*, 1976).

Details of the assay for determining the binding capacity of testicular protein to hCG was described previously (Bedrak, *et al.*, 1980).

Responsivity of the intact rat to exogenous GnRH or hCG stimulation

The experimental protocol for the assessments of response to GnRH (courtesy of Dr. R. Geiger, Farbwerke Hoechstag, Germany) is briefly described. The rat was anesthetized between 09:00 to 11:00 and approximately 1.0 ml of blood sample was withdrawn from the heart (for determination of base level of hormones). Immediately thereafter GnRH (50 ng/100 g body weight) was administered, intracardiacly, in a volume of 0.5–1.0 ml. Twenty-five minutes later the second blood sample was withdrawn (for determining the treatment effect on the concentration of hormones). Thus each rat served as its own control. Serum was separated as described above and the concentration of LH, FSH and T was determined by RIA. To assess the response of the rat to exogenous gonadotrophin, the rats were injected subcutaneously with 3IU hCG/100 g body weight (0.5 ml) for 3 consecutive days between 09:00 to 11:00. Control rats were injected with the vehicle (saline) on a comparable time schedule. The rats were killed 24h after the last injection and the serum was saved for estimation of T concentration.

Determination of hypothalamic content of GnRH

The extraction procedure of GnRH from the hypothalamus and its determination was carried out according to the method of Koch and Baram (1976).

Determination of FSH and LH

The concentration of these hormones was measured by double-antibody RIA (Daane and Parlow, 1971) using kits supplied by the National Institute of Arthritis, Metabolic and Digestive Diseases (NIAMDD). The hormones were assayed in duplicate and in two dilutions and the results were expressed in terms of their NIAMDD rat RP-1 reference preparations.

Statistical analysis

The data were statistically analyzed using the Student's t-test. The results were considered significantly different when the variation between the experimental groups differed at $p < 0.05$.

RESULTS

The effect of aging on GnRH content in the medial basal hypothalamus

Determination of GnRH content in the hypothalamus disclosed that aging was accompanied by a decrease ($p < 0.01$) in GnRH. Namely, the content of GnRH in the young and the old rat were 7.0 ± 0.38 and 4.3 ± 0.28 ng/hypothalamus, respectively.

Responsivity of the intact rat to a stimulus of GnRH or hCG

The increase in serum level of FSH, LH and T following intracardical injection of GnRH was significantly lower ($p < 0.01$) in the old rat than that of the young (Fig. 1 – A).

The data are presented as the percentage increase in the concentration of the respective hormone over the base level observed prior to treatment. Similarly, the percentage increase in serum T concentration following subcutaneous injections of hCG was significantly lower ($p < 0.01$) in the old rat than that of the young one (Fig. 1 – B). Here the data are presented as percentage increase in serum T concentration caused by the hCG treatment, over the level observed in the control (vehicle treated rats).

Binding of ^{125}I-hCG to testicular protein of rats of different age groups

Testicular protein exhibited specific binding of ^{125}I-hCG with association constant (Ka) of about $10^{10}M^{-1}$ based on a molecular weight of 36,000 daltons (Fig. 2). The number of binding sites (receptors) per mg protein was 4,200 ± 800 at the age of 1 month, gradually increased to a zenith of 7,900 ± 500 receptors/mg protein ($p < 0.01$) at the age of 3–4 months and decreased gradually thereafter to a nadir of 1,100 ± 350 ($p < 0.01$) at the age of 18 and 24 months (Fig. 3).

Binding of ^{125}I-hCG to the Leydig cell isolated from testes of young (3–4 month-old) and old (24 month-old) rats

Leydig cells of young and old rats exhibited a similar specific binding of ^{125}I-hCG, with an apparent Ka of $10^{10}M^{-1}$, based on a molecular weight of 36,000 daltons (Table 1). The Leydig cells isolated from the old rat showed a consistent lower number of receptors than the young. However, the absolute number of receptors per Leydig cell varied with the different hCG iodinated preparations. Therefore the degree of experimental variance for assays, done with different hCG iodinated preparations, did not allow for absolute comparison of binding capacity from all experiments. Thus, the results of the number of receptors per Leydig cell isolated from the old rat was expressed as a percentage of the number of receptors per Leydig cell isolated from the young rat in the same experiment.

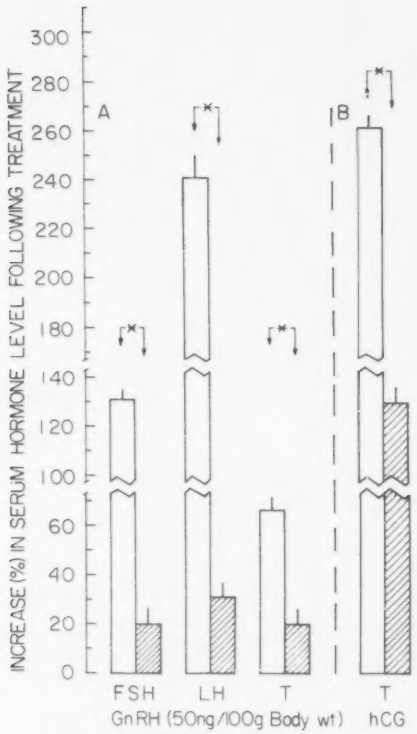

FIG. 1. Responsivity of the intact rat to exogenous hormonal treatment. A—a single intracardiac injection of GnRH. Blood samples were taken before and 25 min following injection. Data are shown as percentage increase in hormone concentration from basal level (observed prior to treatment) to that observed 25 min post-treatment (mean ± SEM of 9–15 rat/group). B—subcutaneous injections of hCG (3IU/100 g body wt for 3 days). Blood samples were taken 24 h following last injection. Data are shown as percentage increase in T concentration from control (injected with vehicle on the same time schedule) and hCG treated animals (mean ± SEM of 10–15 rats/group). Empty column, young rats; striped column, old rats (significantly lower [$p < 0.01$] than the young rats).

These pooled data indicate that the number of receptors for hCG in the Leydig cell of the old rat is 72 ± 3.0% of the number of receptors found in the young rat ($p < 0.01$, Table 1).

Production of T and c-AMP by the isolated Leydig cell challenged with hCG

The responsivity of the Leydig cell, isolated from testes of young and old rats, to a challenge of hCG is summarized in Table 1. These data clearly demonstrate that the production rate of T by Leydig cell isolated from the old rat was significantly lower ($p < 0.01$) than that of the young. On the other hand, no statistical differences were observed between the Leydig cells of the two age groups to synthesize c-AMP following a challenge of hCG (Table 1). These data suggest that aging is associated with aberration in

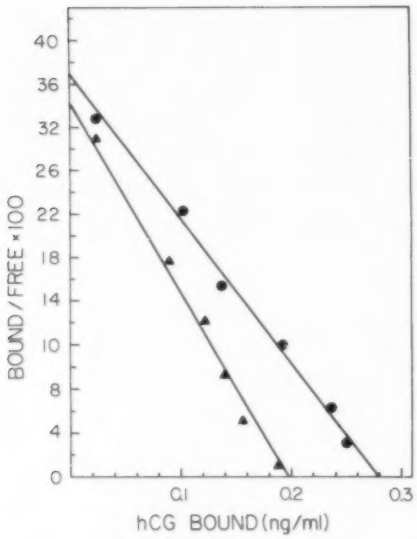

FIG. 2. Scatchard-plot analysis of ^{125}I-hCG binding to testicular protein. Protein suspension (0.1 ml, 100–150 μg) were incubated in triplicate with ^{125}I-hCG and varying amounts of unlabeled hCG at 34.5°C for 90 min. Each line is derived from incubation of aliquots of protein suspension obtained from 2–3 rats. The number of binding sites per mg protein, obtained from the intercept of the lines with the abscissa was 7.31×10^9 and 3.89×10^9 for the 3-month (●) and the 13-month (▲) old rat, respectively.

the activity of the steroidogenic enzymes associated with T production and not with alteration in the function of adenylate cyclase.

DISCUSSION

The present study brings evidence to support the thesis that aging is coupled with alteration in the function of all levels of control of testicular activity (hypothalamus, pituitary and the Leydig cell) which eventually lead to lower concentration of circulating hormones and poor reproductive capacity. With respect to the hypothalamus, the data presented herein indicate that the hypothalamic content of GnRH in the old rat is significantly lower ($p < 0.01$) than that of the young. These data are in accord with the findings of Riegle and Meites (1976) and Riegle, Meites, Miller and Wood, (1977) who demonstrated that the old rat has a lower capacity, than the young one, to secrete GnRH. These data then, strongly support the contention that the fundamental process of synthesis and release of GnRH, which is imperative for adequate function of the pituitary, is impeded with aging. Moreover, the significantly lower response of the intact rat to a stimulus of GnRH suggests that the second level of control for testicular activity, namely the pituitary gland, is also abrogated with aging. Our data, which are in accord with others (Riegle and Meites, 1976; Riegle, *et al.*, 1977), clearly demonstrate that the percentage increase in the old rat; over base level; of LH, FSH and T was significantly lower ($p < 0.01$) than that of the young. These findings could be explained, in part, by a decline in the biosynthetic capacity of adenohypophysis and/or a decrease in pituitary content of LH and FSH characterizing

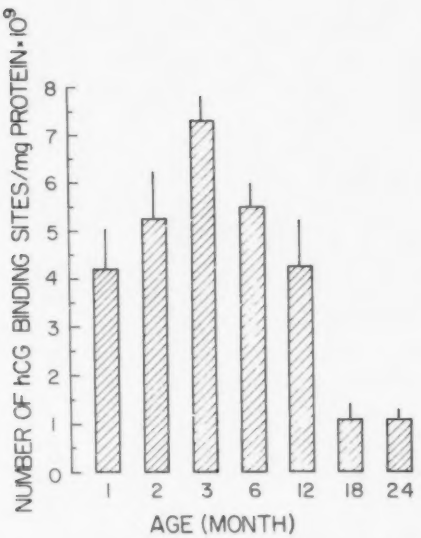

FIG. 3. Concentration of hCG binding sites in testicular protein of 1–24 months-old rats. The number of binding sites was obtained by the Scatchard-plot analysis, as described in the section of Material and Methods, using 8–10 concentrations of hCG in each experiment. Values are means ± SEM of 2–4 experiments. The concentration of binding sites at 3 months of age was significantly higher ($p < 0.01$) than other age groups, however, the differences among 1, 2, 6 and 12 months or between 18 and 24 months of age was not significant. The concentration of binding sites observed in 18 and 24 months-old rats was significantly lower ($p < 0.01$) than other age groups.

the old rat (Clemens and Meites, 1971; Bruni, Huang, Marshall and Meites, 1977; Bethea and Walker, 1979) which undoubtedly restrict the gonadotrophins' pool available for release.

Aging is also associated with aberration in the function of the last component of the

TABLE 1. CHARACTERISTICS AND ACTIVITY OF ISOLATED LEYDIG CELLS OF YOUNG AND OLD RATS: RELATIVE NUMBER OF hCG BINDING SITES (RECEPTORS), SPECIFIC BINDING OF hCG (Ka) AND RESPONSIVITY TO AN hCG CHALLENGE (PERCENT INCREASE, FROM BASE LEVEL, IN THE PRODUCTION OF T AND c-AMP).

Rats	Relative receptor number (%)[1]	Ka	Percent increase from base level[2]	
			T	c-AMP
Young	100 ± 5.2	1.06 ± 0.26 × $10^{10}M^{-1}$	325 ± 6.5	650 ± 22.4
Old	72 ± 3.0*	1.56 ± 0.21 × $10^{10}M^{-1}$	86 ± 14.6*	566 ± 65.3

[1]Number of receptors in Leydig cells of old rats is expressed as percent of receptors in Leydig cell isolated from young rats assayed with different ^{125}I-hCG preparations (mean ± SEM of 5 experiments).

[2]Production rate of T and c-AMP by the Leydig cell challenged with hCG. Cell suspensions ($1 - 3 \times 10^5$ living cells) were incubated with the appropriate buffer (for details see Materials and Methods) fortified with 10 ng hCG/ml. Data expressed as percent increase from controls in which the hCG was replaced by the vehicle (means ± SEM of 5 experiments).

*Significantly lower ($p < 0.01$) than the young rat.

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axis; i.e., the testes; to synthesize T irrespective of the circulating levels of LH. This suggestion is based on the observation that exposure of the intact old rats to a prolonged stimulus (72 h) of hCG lead to a significantly lower ($p < 0.01$) rise in serum T concentration than that observed in the young (Fig. 1 – B). This characteristic response of the old rat is consistent with other reports in the rat (Miller and Riegle, 1978; Kaler and Neavens, 1981) and in man (Rubens, *et al.*, 1974). These findings, therefore, negate the thesis suggesting that since the old rat appears to have a deficiency in circulating LH, the functional defect of the Leydig cell merely represents a condition of chronic understimulation (Miller and Riegle, 1978; Harman, Danner and Roth, 1978). One of the possible explanations for this phenomenon could be associated with the alteration in the nature and function of the gonadotrophin receptors in the testes of the old animals as it is reflected on the one hand by a significantly lower number of hCG receptors/mg testicular protein of the old rat ($p < 0.01$, Figs. 2 and 3) and on the other hand by a comparable Ka ($10^{10}M^{-1}$) value to that of the young rat.

Similar results were observed when the isolated Leydig cells were challenged with hCG, *in vitro*. That is to say, T production rate by the Leydig cell of the old rat was significantly lower ($p < 0.01$) than that of the young along side a lower number of hCG receptors (Table 1). These phenomena were not accompanied by a significant change in activity of adenylate cyclase (Table 1). The alterations in the Ka for hCG receptors in the Leydig cell isolated from the old ($1.56 \pm 0.21 \times 10^{10}M^{-1}$) and the young rat ($1.06 \pm 0.26 \times 10^{10}M^{-1}$) were not statistically significant. That the Leydig cell of the old rat secreted about one-third of the level produced by the young rat was reported in an earlier abstract by Pirke, Geiss and Vogt (1978) in which no reference was made to c-AMP production and receptor number. A later report by Tsitouras, *et al.* (1979) confirmed the observation that the Leydig cell of the old rat had a significantly lower capacity to synthesize T and also demonstrated a lower number of hCG receptor than the young one. The discrepancy between the evidence presented herein and those described by Bethea and Walker (1979) probably stems from the fact that the latter investigators conducted their research with the Fisher 344 strain of rats which develops interstitial cell adenomas with age.

Tsitouras, *et al.* (1979) demonstrated a significant alteration ($p < 0.01$) in the Ka for hCG receptors in the Leydig cells isolated from the old rat. They proposed that these changes in the Ka values represent basic differences in the cell membranes and/or in the structure of the receptor molecule itself. Though the differences in the Ka values, between the old and the young rat, obtained in our study were not statistically significant, the trend of change resembled the finding of Tsitouras, *et al.* (1979). Thus further research is needed to elucidate the nature of alteration which takes place in the structure/function of the receptor molecule during aging.

It is rather doubtful that the functional defect of the Leydig cell isolated from the old rat stems primarily from the decline in the number of LH receptors. This thought is based on the following argument: Maximal stimulation for T production by the Leydig cell is achieved when less than 20% of the LH receptors are saturated (Catt and Dufau, 1975); the magnitude in T production rate by the Leydig cell isolated from the old rat challenged with hCG as demonstrated in our study, is significantly lower than the decline in the number of LH receptors noted in the cell; the vast differences in T production rate noted in our study between Leydig cells isolated from the young and old rat, when challenged with hCG, is independent of alteration in the activity of adenylate cyclase. In view of these facts it is suggested that aging provokes aberrations in the function of the Leydig cells

which lie distal to the binding of the trophic hormone to the cellular membrane and the adenylate cyclase steps. Some of these phenomena are inherent to alteration in the activity of several stercidogenic enzymes associated with T production and catabolism as was suggested by the observation of Collins, Bell and Tsang, (1972). This observation is, no doubt, associated with the decline in activity of the 5-en-3β hydroxysteroid dehydrogenase and 5-en-isomerase (3β-ol system) which accompanies the process of aging in the rat (Leathem and Albrecht, 1974). It appears, therefore that in the Leydig cell of the old rat, the depressed activity of the 3β-ol system and the concomitant enhanced 7α hydroxylation of T (Chan, *et al.*, 1977) render an overall low output of the active androgen. It stands to reason that the responsiveness of the old rat to hCG administration, as measured by serum T, is a result of a partial restoration in the activity of some of the enzymes involved in the androgen biosynthesis (Leathem and Albrecht, 1974; Miller and Riegle, 1975; Chan, *et al.*, 1977; Chap, 1980; Chan, *et al.*, 1981).

In summary, the data presented herein suggest that the phenomenon of senescence of the male rat is due to alteration in the function and activity of all components of the hypothalamo-pituitary-testicular axis which account for the reduced sexual arouse and loss of fecundity.

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SUMMARY

Male Sprague-Dawley-derived rats, aged 3–4 months (young) and 24 months (old), were investigated as to the integrity of the hypothalamic-pituitary-testicular axis (axis) with respect to testosterone (T) production. The results of this study clearly demonstrate that aging reduces ($p < 0.01$) the hypothalamic content of gonadotrophin releasing hormone (GnRH), decreases the capacity of the pituitary ($p < 0.01$) to synthesize and or release follicle stimulating hormone and luteinizing hormone (LH) following a single stimulation of GnRH (50 ng/100 g body weight), lowers the capacity of the testes to produce T ($p < 0.01$) following multiple subcutaneous injections of human chorionic gonadotrophin (hCG 3IU/100 g body weight for 3 consecutive days), decreases the number of Leydig cell LH receptors, increases slightly the association constant (Ka) of ^{125}I-hCG to the Leydig cell (Ka = $1.56 \pm 0.21 \times 10^{10} M^{-1}$, $1.06 \pm 0.26 \times 10^{10} M^{-1}$ for the old and young rats, respectively) and decreases the *in vitro* responsiveness of the hCG-challenged Leydig cell to synthesize T ($p < 0.01$). These phenomena are independent of a major alteration in the capacity of the hCG challenged Leydig cell to produce adenosine 3′, 5′-monophosphate. It is concluded that the decline in testicular activity accompanying senescence is not inherent to the testes only but is also associated with alteration in the function of the hypothalamus and pituitary which eventually lead to the loss of fecundity.

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REDUCTION OF LIPOFUSCIN BY CENTROPHENOXINE AND CHLORPROMAZINE IN THE NEURONS OF RAT CEREBRAL HEMISPHERE IN PRIMARY CULTURE

R. OHTANI and S. KAWASHIMA*

Zoological Institute, Faculty of Science, University of Tokyo, Tokyo 113 and
Zoological Institute, Faculty of Science, Hiroshima University, Hiroshima 730, Japan

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Abstract—Cells from neonatal rat cerebral hemispheres were dispersed by trypsin and cultured for 32 days. Histochemical, fluorescence, and electron microscopic analyses demonstrated that lipofuscin pigments increased in neuronal and non-neuronal cells in primary culture according to the lapse of time. When centrophenoxine (10^{-4} or 5×10^{-4} M) or chlorpromazine (10^{-6} or 10^{-5} M) was added to the medium, the accumulation of lipofuscin pigments in neurons was significantly reduced. However, the effects of these agents were not detected in non-neuronal cells.

INTRODUCTION

THE ACCUMULATION of lipofuscin is a consistent age-related cytological alteration in a variety of post-mitotic cells in mammals. The lipofuscin pigments have been identified by their autofluorescence, histochemical reactions and electron microscopic profiles in neurons of aging rodents (Hasan and Glees, 1972; Hasan, Glees and El-Ghazzawi, 1974a; Spoerri and Glees, 1975; Davies and Fotheringham, 1981). The same criteria have also been used for the identification of lipofuscin pigments in cultures of rat dorsal root ganglion cells (Spoerri and Glees, 1973) and neuroblastoma cells (Nandy and Schneider, 1976). According to Schneider and Nandy (1977) the pigment accumulation in cultures of neuroblastoma cells was enhanced by treatment with papaverin or prostaglandin E_1, while it was reduced by centrophenoxine. The *in vivo* effects of centrophenoxine seem to be well so established that it reduces the rate of lipofuscin formation in neurons of aged mice, rats and guinea pigs (Nandy, 1968; Nandy and Bourne, 1966; Spoerri and Glees, 1975; Hasan, *et al.*, 1974 a,b; Riga and Riga, 1974). Reduction of lipofuscin was also reported in the neurons of 20-month-old mice receiving prolonged treatment with chlorpromazine (Samorajski and Rolsten, 1976).

As Schneider and Nandy (1977) have already pointed out, the cell culture system provides a useful model for the study of effects of these drugs on the cellular aging. The present

*All correspondence and requests for reprints should be addressed to Dr. Seiichiro Kawashima, Zoological Institute, Faculty of Science, Hiroshima University, Hiroshima City 730, Japan.

paper will deal with the effects of centrophenoxine (CP) and chlorpromazine (CPZ) on the rate of lipofuscin pigment formation in the cells of rat cerebral hemispheres in primary culture.

METHODS AND PROCEDURES

Preparation of cell suspension

Sprague-Dawley/Tw rats within 48 hr. after birth were killed by decapitation, and the cerebral hemispheres were aseptically taken out and pooled in the standard medium. After the pooled tissue was minced, the standard medium was replaced by Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 0.25% trypsin and incubated at 37°C for 10 min. The aliquots of cell suspension of the first trypsinization were discarded, and fresh trypsin solution was added. Dissociated cells after gentle pipetting and incubation were transferred to a flask containing a large amount of the standard medium. Several harvests of cells were obtained in the same way until only a fibrous material was left. The pooled cell suspensions were filtered through a nylon cloth and centrifuged at 1000 r.p.m. for 5 min. The resultant cell pellet was resuspended in the standard medium. The number of viable cells (0.1% trypan blue exclusion test) was counted by means of a hemocytometer. The cell suspension was diluted at the final concentration of 1.2×10^6 cells/ml.

Culture vessels

On the sterile coverslips placed in each well of multiwell tissue culture plate (Falcon 3008), 0.1 ml of cell suspension was added. The culture plates were incubated at 37°C in an atmosphere of 95% air–5% CO_2. On the following day, 0.4 ml of the standard medium was added to each well to make the final concentration of cells at 2.4×10^5/ml. The medium was renewed on the 5th and 8th days of culture and after that the medium with or without centrophenoxine (CP) or chlorpromazine (CPZ) was renewed every two days. The day on which the chemicals were first added (7 days of cultivation) was designated as Day 0. Cultures given no chemicals served as controls.

Medium and chemicals

Standard medium consisted of Eagle's minimum essential medium supplemented with 16% horse serum, 0.7% glucose, 2 mM glutamine, 20 mM Na-bicarbonate, 100 IU/ml penicillin and 100 μg/ml fungison. CP and CPZ were dissolved in the standard medium at the final concentrations of 10^{-4} M or 5×10^{-4} M, and 10^{-6} M or 10^{-5} M, respectively.

Histochemical and electron microscopic studies

For the demonstration of the lipofuscin pigments in cells, the coverslips were either stained with periodic acid-Schiff (PAS) reagent or with lead phosphate acid phosphatase methods (Nandy, 1968, 1971; Gomori, 1950) or exposed to ultra-violet light at a wavelength of 350–500 nm and an emission range of more than 550 nm for autofluorescence analysis. On Days 0, 5, 15 and 25, the numbers of cells containing the lipofuscin pigments which showed intense stainability in PAS, strong acid phosphatase activity or characteristic yellow autofluorescence were counted at ten different fields (a total of 9.8×10^5 μM^2) in each coverslip with the aid of an ocular grid micrometer. Three coverslips in each concentration of chemicals and 5 coverslips in matched control groups were studied simultaneously at each termination of culture. The neurons and other types of cells (mainly consisting of glial cells and fibroblasts) were separately counted. The neurons were identified by their morphological characteristics in modified Bodian's preparations of other coverslips (Otsuka, 1962). The neurons were usually overlaying the monolayer of neuroglial and fibroblastic cells, and showed a few processes with irregular varicosities and thickenings or one or two smooth long processes.

For electron microscopy, the cells on the coverslips were fixed on Days 0 and 25 in 2% glutaraldehyde and 3% paraformaldehyde for 1 hr., then cell layers were gently peeled off from the coverslips and post-fixed in 1% osmium tetroxide for 1 hr. Both fixatives were buffered with 0.1 M cacodylate at pH 7.4. After the fixation, the cells were dehydrated with graded series of ethanol and embedded in TAAB resin. Ultrathin sections cut on a Porter-Blum MT2-B ultramicrotome were stained with uranyl acetate and lead citrate. They were examined with a JEM-100 C electron microscope.

RESULTS

Autofluorescence and histochemical analyses demonstrated a marked increase in the lipofuscin pigments in both neuronal and non-neuronal cells according to the passage of

time in untreated control cultures. A characteristic yellow autofluorescence emitted by ultra-violet light is a reliable criterion for the presence of lipofuscin. In Day 0 cultures a small amount of autofluorescent pigments was observed (Fig. 1a), however, the pigments increased markedly in Day 25 cultures (32 days of cultivation, Fig. 1b). Stainability to PAS and acid phosphatase activity also increased in Day 25 cultures (Figs. 2b and 3b) as compared with Day 0 cultures (Figs. 2a and 3a).

Histoquantitative data exhibited a steady increase in the percentage of cells showing autofluorescence during the 25-day period (Figs. 4 and 5). Neurons bearing autofluorescent pigments were 16.2% of all the neurons observed on Day 0 and the incidence was increased to 52.7% on Day 25 (Fig. 4). The autofluorescence was found in 16.9% of non-neuronal cells on Day 0 and 34.3% on Day 25 (Fig. 5). The differences in the percentage of the autofluorescent cells between Day 0 and Day 25 were statistically significant ($p < 0.001$ for the neurons and $p < 0.01$ for the non-neuronal cells, Fisher's exact probability test). Incidences of PAS-positive cells and the cells showing strong acid phosphatase activity were also significantly increased as the culture period was prolonged. The effects of 10^{-4} M and 5×10^{-4} M CP and 10^{-6} M and 10^{-5} M CPZ in the medium on the incidence of cells containing lipofuscin pigments are summarized in Figs. 4 and 5. Both CP and CPZ significantly reduced the percentage of neurons bearing lipofuscin pigments (Fig. 4). There were no differences in the degree of reduction among neurons given either 10^{-4} M or 5×10^{-4} M CP, or 10^{-5} M CPZ, on the basis of autofluorescence and histochemical analyses. The treatment with 10^{-6} M CPZ also reduced the pigments in autofluorescence and PAS preparations, however, it failed to affect significantly on acid phosphatase activity on Days 15 and 25 (Fig. 4). In non-neuronal cells the effects of CP and CPZ were less marked than those in neurons, evidence of reduction in pigment accumulation being not obtained by autofluorescence and acid phosphatase activity (Fig. 5).

In electron microscopy lipofuscin pigments appeared as varying shapes of electron dense bodies. The number of pigments increased in neuronal cytoplasm on Day 25 (Fig. 6a) as compared with that on Day 0. Treatment with CP or CPZ for 25 days inhibited the formation of lipofuscin pigments in the neurons (Fig. 6b). In the glial cells, the number of pigments was less than that in the neurons (Fig. 7a). Neither CP nor CPZ showed a significant effect on the number of pigments (Fig. 7b).

DISCUSSION

The present study in the primary culture showed that the neurons as well as the non-neuronal cells from the rat cerebral hemisphere accumulated intracellularly lipofuscin pigments according to the lapse of time. The pigments were similar to those of neurons in aged animals on the basis of autofluorescence and histochemical reactions. In the culture system, however, the rate of pigment formation was faster than that of the *in vivo* system. This study also showed that CP and CPZ treatments were effective in reducing the rate of the pigment formation in the cultured neurons. The effects of CP on the pigment formation were in good accord with those reported by Schneider and Nandy (1977) in mouse neuroblastoma cells in culture. Their results and the present ones were consistent with the earlier reports on the neurons of aged mice, rats and guinea pigs treated with CP (Nandy, 1968; Nandy and Bourne, 1966).

In the present study, however, the effects of CP and CPZ were not marked on the non-neuronal cells. This may be caused, at least partly, by a mixed population of dividing and non-dividing cells consisting of glial cells, fibroblasts and endothelial cells. The dif-

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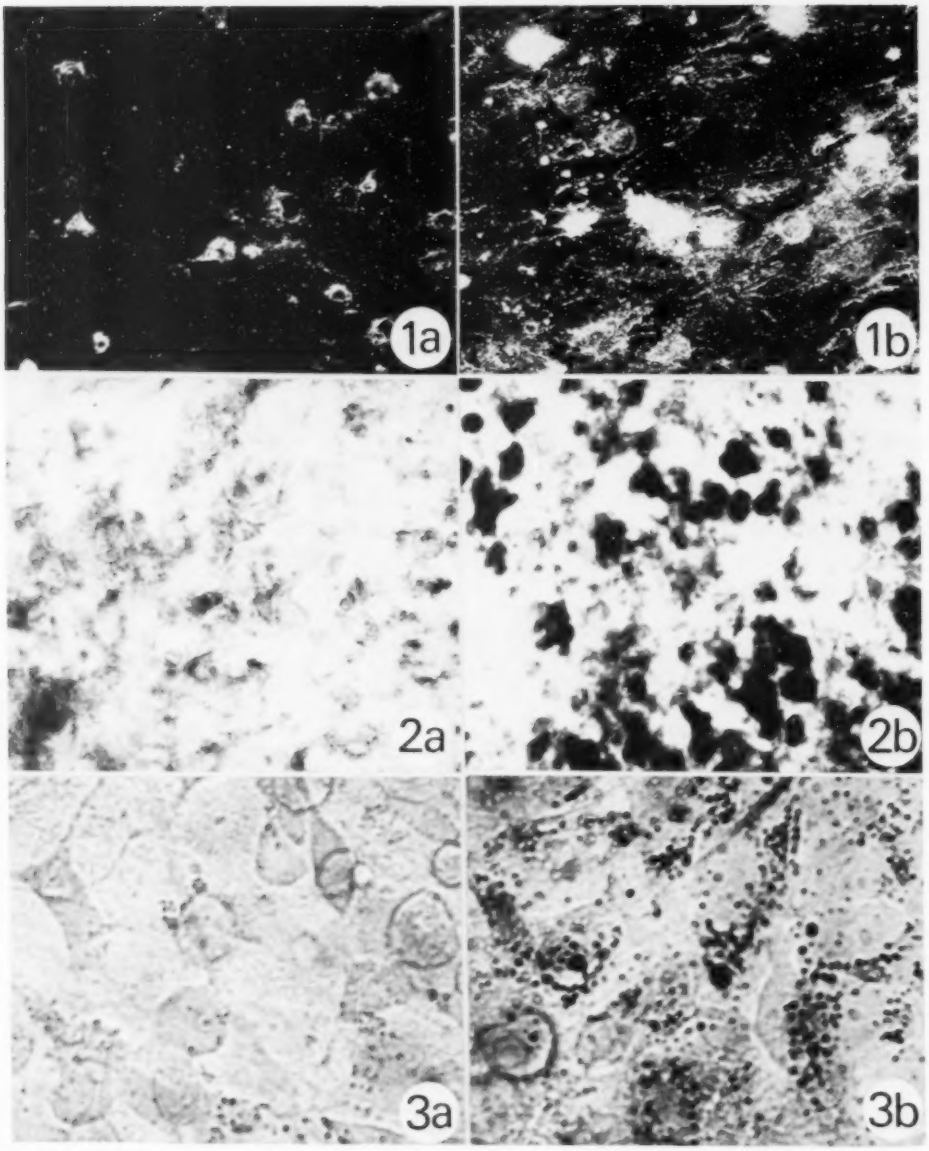

FIG. 1. Autofluorescent cells on Day 0 (la) and Day 25 (1b) cultured in standard medium (× 550).

FIG. 2. Periodic acid-Schiff positive cells on Day 0 (2a) and a marked increase on Day 25 (2b) cultured in standard medium (× 530).

FIG. 3. Deposition showing acid phosphatase activity which was very weak on Day 0 (3a) and increased markedly on Day 25 (3b) in control cultures (× 800).

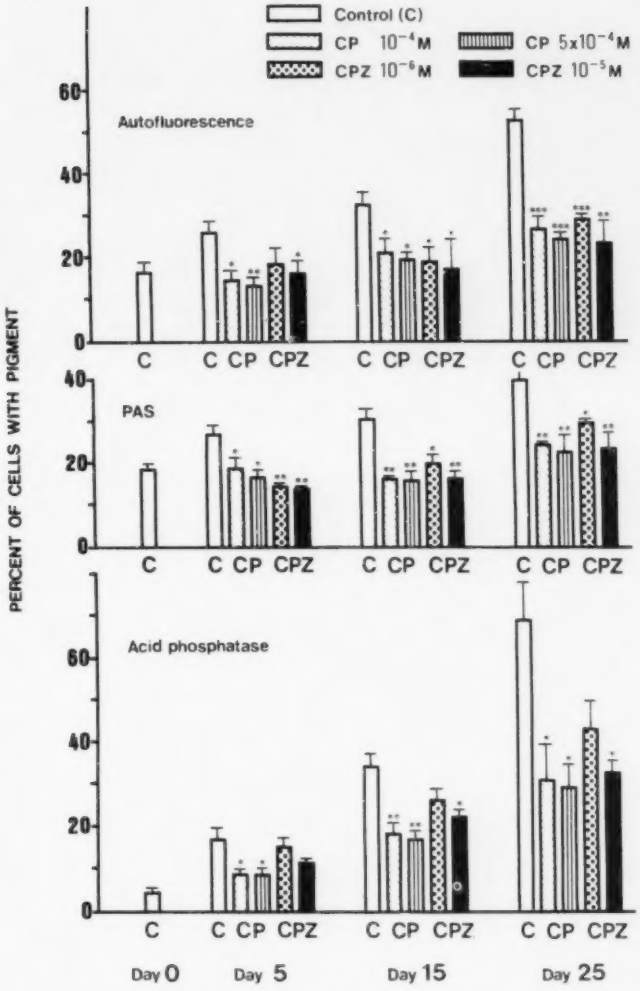

FIG. 4. Reduction in the rate of lipofuscin pigment formation by 10^{-4} M and 5×10^{-4} M centrophenoxine and 10^{-6} M and 10^{-5} M chlorpromazine in neurons from rat cerebral hemisphere. Bars depict the standard errors of the means. Differences from matched controls: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

ficulties in identifying these cell types separately should be overcome in future studies of the primary culture system.

The phagocytotic function of some constituent cells may contribute to the distribution and rate of accumulation of the lipofuscin pigments in primary culture cells. Considerable dissolution and removal of the pigments in neurons by phagocytic cells were reported by Spoerri and Glees (1975) in the anterior hypothalamus of senile pigs.

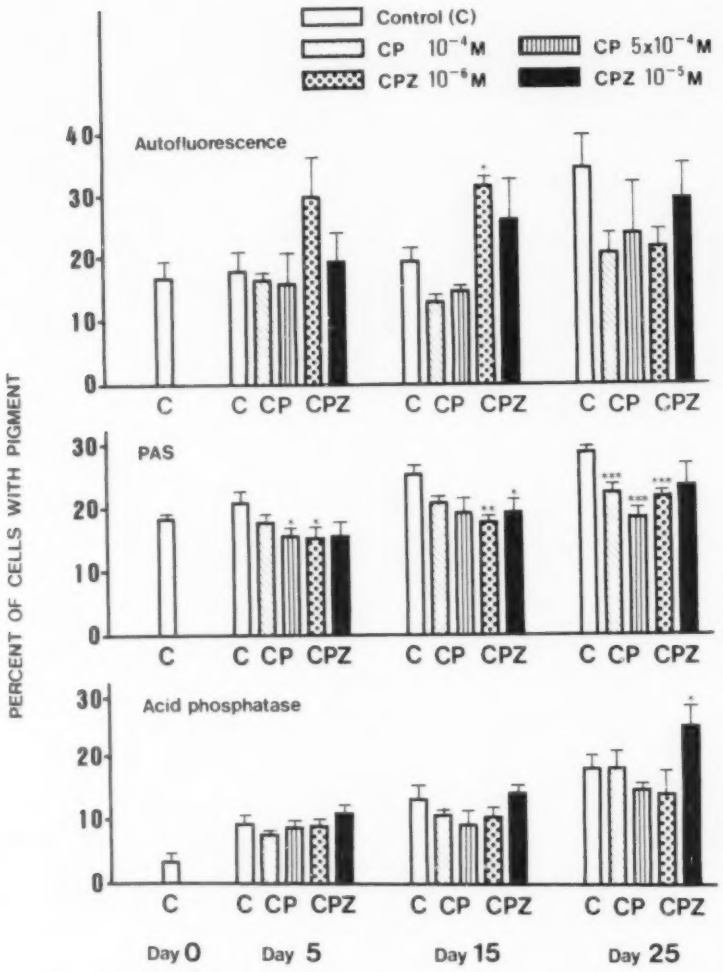

FIG. 5. Failure of 10^{-4} M and 5×10^{-4} M centrophenoxine and 10^{-6} M and 10^{-5} M chlorpromazine to induce consistent reduction in the lipofuscin pigment formation in non-neuronal cells from rat cerebral hemisphere.

Samorajski and Rolsten (1976) reported the effects of chronic administration of CPZ on reducing the lipofuscin pigment formation in the neurons of the nucleus reticularis gigantocellularis in mice, and they suggested that CPZ acted through molecular changes in cellular membrane. In the present study, CPZ was also effective on the neurons in culture, but its effect was less pronounced in non-neuronal cells. Nevertheless, the exact mechanism of CP and CPZ on reducing lipofuscin is not clear at present. In conclusion, the central nervous cells in primary culture provides a good model to examine the cellular mechanisms involved in the lipofuscin formation and its modification by pharmacological agents.

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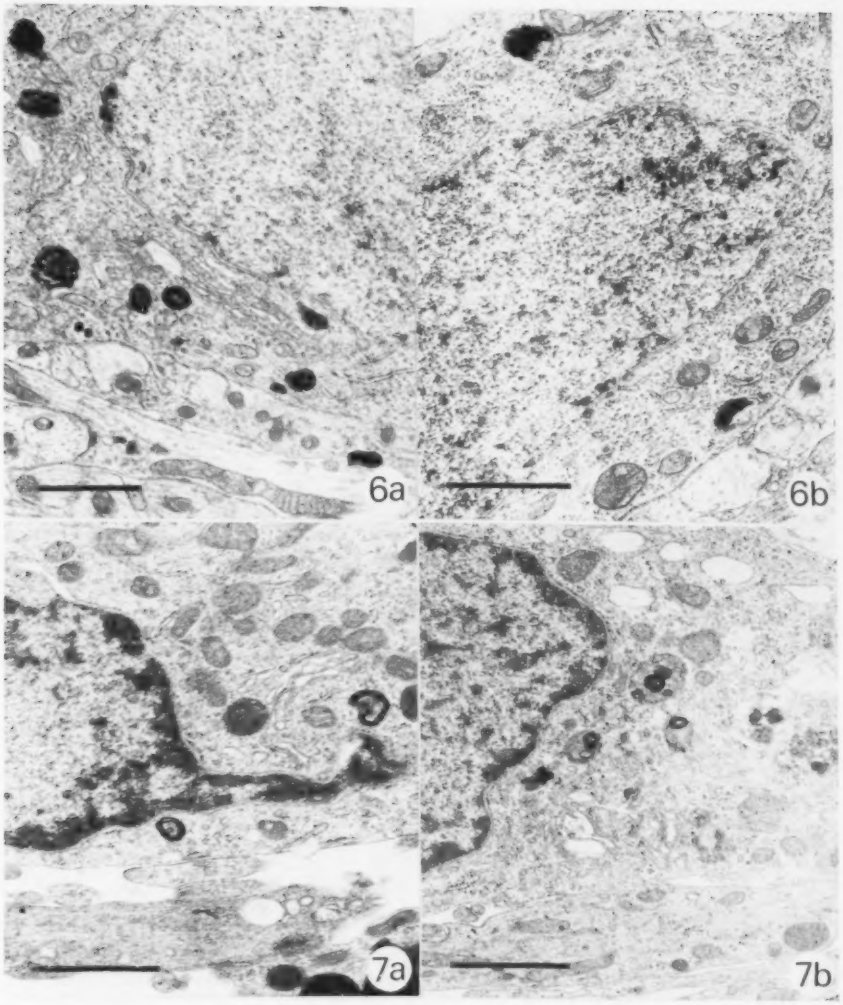

FIG. 6. Electron micrographs of the neurons on Day 25 of culture. 6a: untreated, 6b: 10^{-4} M centrophenoxine-treated. Note the reduction in number of lipofuscin pigments. Scale: 1 μM.

FIG. 7. Electron micrographs of glial cells on Day 25 of culture. 7a: untreated. 7b: 10^{-4} M centrophenoxine-treated. Scale: 1 μM.

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THE EFFECTS OF ENFORCED RUNNING ON THE GASTROCNEMIUS MUSCLE IN AGING MICE:

An Ultrastructural Study

RUTH LUDATSCHER,* MICHAEL SILBERMANN,**
DAVID GERSHON,† and ABRAHAM REZNICK†

Departments of Pathology* and Morphological Sciences,** Faculty of Medicine and the Department of Biology,† Technion – Israel Institute of Technology, Haifa, Israel

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Abstract—C57BL/6J mice (6, 19 and 27 months old) were trained in an electrically driven treadmill for a total period of 10 weeks. The training program started with 5 min. run per day and reached a maximum of 30 min. per day. Light and electron microscopy examinations of the gastrocnemius muscle showed that in young animals endurance training did not evoke any appreciable changes. In contrast, both test animals and their controls in the old group revealed clear signs of muscle cell atrophy which for the most part was accompanied by focal loss of myofilaments, mitochondrial changes and an increase in the amount of endomyseal collagen fibrils. In the old-trained group, the most obvious abnormality comprised of multiplication of both the T-tubules and of the tubules of the sarcoplasmic reticulum. Consequently, most of these animals showed the formation of tubular aggregates in their gastrocnemius muscle. Hence, it appears that endurance training in old animals tends to enhance the regressive changes that accompany the aging process of mammalian skeletal muscle.

INTRODUCTION

FOR A LONG TIME studies related to the morphological aspects of aging skeletal muscles have been focused on specimens of insect flight muscles as they contain a specialized system of post mitotic cells demanding high energy requirements (Webb and Tribe, 1974). It became evident, however, that minor differences exist between the basic physiological patterns of insect muscles and those of mammalian skeletal muscles (Peachey, 1968). Recently, valuable data relating to the structure of the skeletal muscle in aging rodents have been provided in Fujisawa (1974, 1975, 1976) and by others (Miquel, Economos, Fleming and Johnson, 1980; Snow, 1977; Tonna and Severson, 1971). De Coster, De Renck, Sieben and Vander Eecken (1981) reported stereological studies on the gastrocnemius muscle of the aging rat, while Tomonaga (1977) described some of the ultrastructural changes that take place in skeletal muscles of the senile human. The purpose of the

Address correspondence and reprint requests to Dr. Michael Silbermann, Laboratory of Biological Structure, National Institute of Dental Research, National Institute of Health, Bldg. #30, Room 211, Bethesda, MD. 20205.

present study was to investigate the ultrastructural features characterizing an aging skeletal muscle that is subjected to an extended period of endurance running.

MATERIALS AND METHODS

A total of 36 female C57BL/6J mice were used in this study. The gastrocnemius muscle of normal mice aged 6, 19 and 27 months was compared with age-matched trained mice. The training program was carried out in an electrically driven treadmill operating at a speed of 8 rpm, and as the treadmill's diameter was 14 cm, the animals' "running" speed was 3.52 m/min. Initially, test animals ran for 5 min/day for a period of one week and thereafter the training time was increased by 5 min/day (for a period of one week), so that by the 6th week the animals reached a daily training time of 30 min. Having attained the above regimen (30 min/day) test animals proceeded with this endurance exercise for an additional period of 4 weeks. Each age group comprised of 6 "runners" and 6 age-matched controls. All mice were killed by neck dislocation and their gastrocnemius muscle was rapidly removed, cut manually into small pieces and immediately immersed in a solution of 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 2 hours. Specimens were then postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for one hour, dehydrated through graded ethanols and embedded in Epon 812. Semithin (1 μM) sections were obtained from 8 different blocks of each muscle, stained with 0.1% toluidine blue and examined with the light microscope. Thin sections (600–800Å) were obtained from at least 3 blocks per animal and were subsequently stained for 8 minutes in saturated aqueous uranyl acetate followed by 10 minutes in 0.2% lead citrate before examination with a Zeiss 9S electron microscope. For each group (test and control) we have examined at least 162 thin sections (3 sections per grid, using 3 different grids per block, at least 3 blocks per animal and 6 animals per group).

RESULTS

Light microscopy

Young animals. The muscle fibers of both test animals and their controls appeared very similar, except for an occasional moderate increase in the size of several fibers in the trained group.

Old animals. Twenty-seven-month-old trained and control animals revealed variations in the size and shape of muscle fibers. Atrophic fibers ranging from 4–12 nm in diameter were frequently encountered either singularly or within small groups comprising of 2–3 cells (Fig. 1). The endomyseal space separating such atrophic cells was occupied by an increased amount of collagenous tissue. Most specimens obtained from the middle-age group (19 months of age) revealed similar features to those noticed in the old group, whereas only in a small number of cases the muscle fibers of the middle-age group did not differ from those of 6-month-old animals.

Electron microscopy

Young animals. A moderate hypertrophy of muscle fibers was evident in the gastrocnemius of trained animal. A similar feature was recently described by Farrar, Martin and Murray-Ardies (1981). In both trained animals and their controls, two types of fibers could be identified at the ultrastructural level: (I) fibers of smaller diameter which for the most part revealed a pronounced Z-line and inhabited aggregates of mitochondria subsarcolemmally; and (II) fibers that could be identified by their larger size which usually demonstrated less mitochondria. Rows of mitochondria were also found along the intermyofibrillar space. Small capillaries were found in close association with the subsarcolemmal accumulations of mitochondria. These kind of fibers most probably

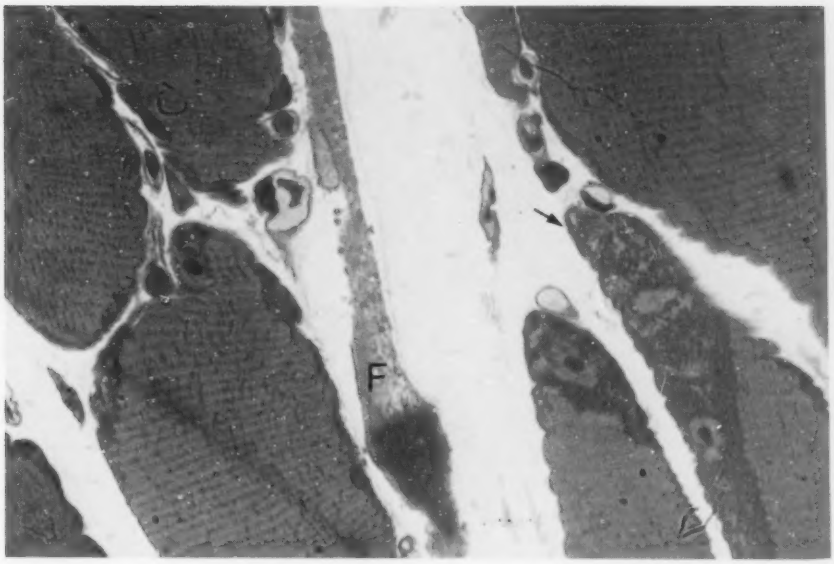

FIG. 1. A section through the gastrocnemius muscle of a 27-month-old mouse that had undergone endurance training for 10 weeks. Note the marked atrophy of one of the fibers in this area (F). Other cells reveal large accumulations of mitochondria at subsarcolemmal sites (arrow). 1 μM section stained with toluidine blue (× 768).

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corresponded to the histochemically defined type I fibers or the red muscle fibers (Mair and Tomé, 1972). In addition, the latter's Z-line was thinner, suggestive of the type II fibers or the white muscle fibers (Mair and Tomé, 1972). Lipofuscin bodies were occasionally encountered in these fibers.

Old non-trained animals. Minor differences were noticed between the 19-months-old and the 27-months-old mice, thus the forthcoming description will correspond to observations made in muscle specimens of both age groups. Variation in the size of muscle fibers was a constant finding, along with the presence of atrophic fibers. Usually, the sarcomere pattern was well preserved (Fig. 2). Lipofuscin bodies were frequent, and were situated near the nucleus or underneath the sarcolemma (Fig. 2). The mitochondria were arranged in rows or in pairs of two, but random accumulations of varied sized mitochondria were also a frequent feature. In some muscle fibers the sarcomeres were disrupted for short segments, the Z-line was dislocated, and smudging of myofilaments occurred in these places (Fig. 3). The tubles of the T-system and the sarcoplasmic tubules were distributed in a regular manner (Figs. 2 & 3). In the gastrocnemius of one 27 month-old mouse, aggregates of elongated tubules were also encountered. Satelite cells were inconspicuous. In the atrophic muscle fibers short chains of nuclei were aligned subsarcolemmally and the nuclei were displaced to the center of the cell.

An increase in the amount of perimysial collagen tissue and sometimes of adipous tissue was noticed in all animals of the aged group. Collagen fibrils adhered to the sarcolemma and sometimes occupied the endomysial space between adjacent muscle fibers.

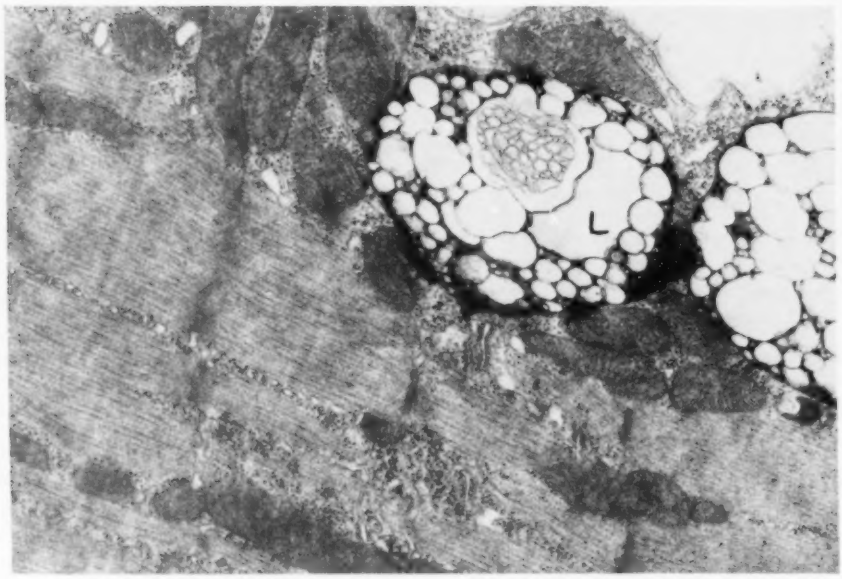

FIG. 2. Electron micrograph from a gastrocnemius of 19-month-old control mouse, showing a regular sarcomere pattern and rows of mitochondria. Lipofuscin bodies (L) are seen underneath the sarcolemma (× 19,000).

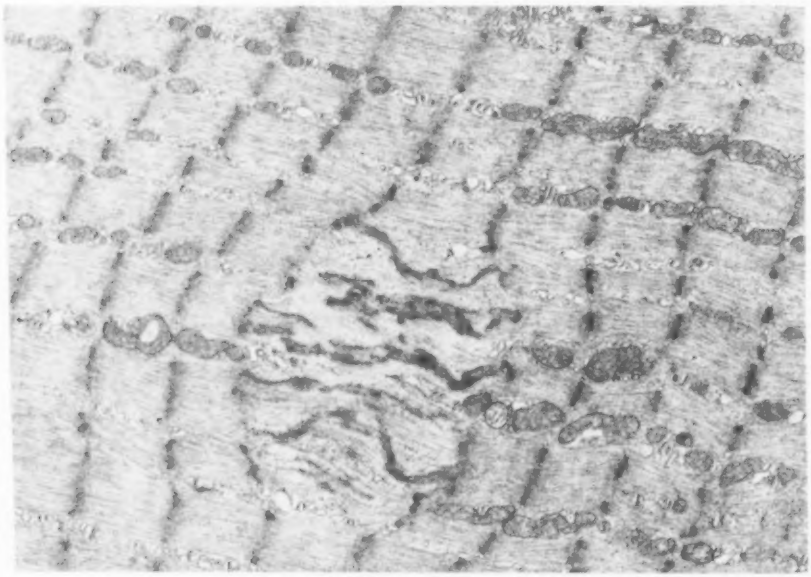

FIG. 3. Muscle in a contracted state from control middle-aged mouse. Note at the central portion of the photomicrograph an area of sarcomeric derangement while degenerated myofilaments appear to fuse together. The T-tubules and those of the sarcoplasmic reticulum are distributed in a normal fashion (× 9,800).

Old trained animals. Numerous lipofuscin bodies were present in all specimens. Significant variation in shape and size of the muscle fibers was recorded. Most atrophic cells averaged a diameter of 10–12 μM, but smaller sizes wre occasionally noted. There were different grades of fiber degeneration in a given section. The myofibrillar changes affected both the atrophic and the normal sized cells. In old animals undergoing endurance training the main alterations could be grouped as follows:

a) *Changes of the Z-line.* In the less affected muscle cells the Z-line was interrupted for only several sarcomeres and was associated with focal disarray of myofilaments. In few cases a massive myofibrillar destruction took place and extended over the entire muscle cell (Fig. 4). Here the Z-line material appeared in zigzag or smeared out, and dense rod bodies occupied the area (Fig. 4 & 5). The rod bodies were of rectangular or of irregular shape (Fig. 5). Chains of subsarcolemmal nuclei were a frequent feature in the myopathic cells (Fig. 4).

b) *Changes in the mitochondria.* Small and large accumulations of mitochondria were noted especially under the sarcolemma (Fig. 4). Giant and bizarre-shaped mitochondria were sometimes seen along with loss or disruption of cristae (Fig. 6). Occasionally lyososomal bodies and lipid vacuoles were seen in the area containing such accumulations of mitochondria.

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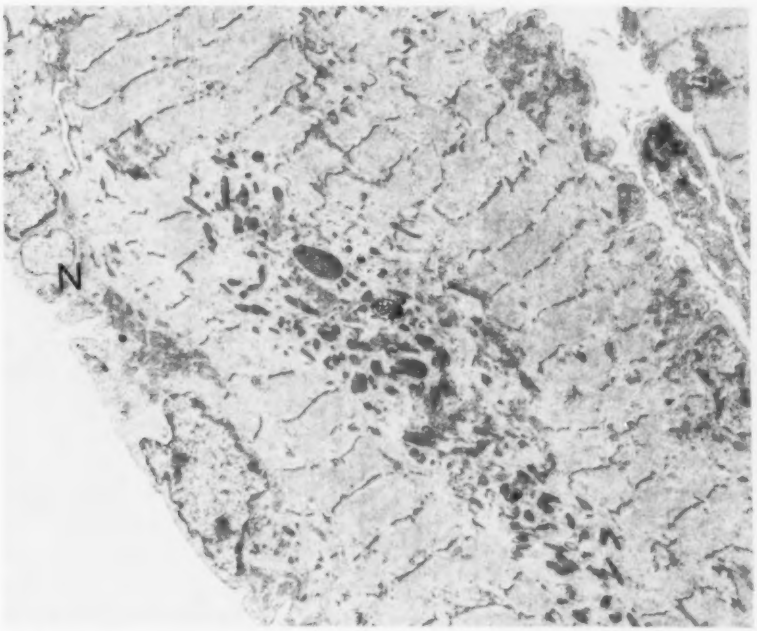

FIG. 4. A section through the gastrocnemius muscle of a 27-month-old trained mouse showing severe myofibrillar changes and numerous rod bodies at the center of the cell. Note the chain of marginal nuclei (N) (× 3,800).

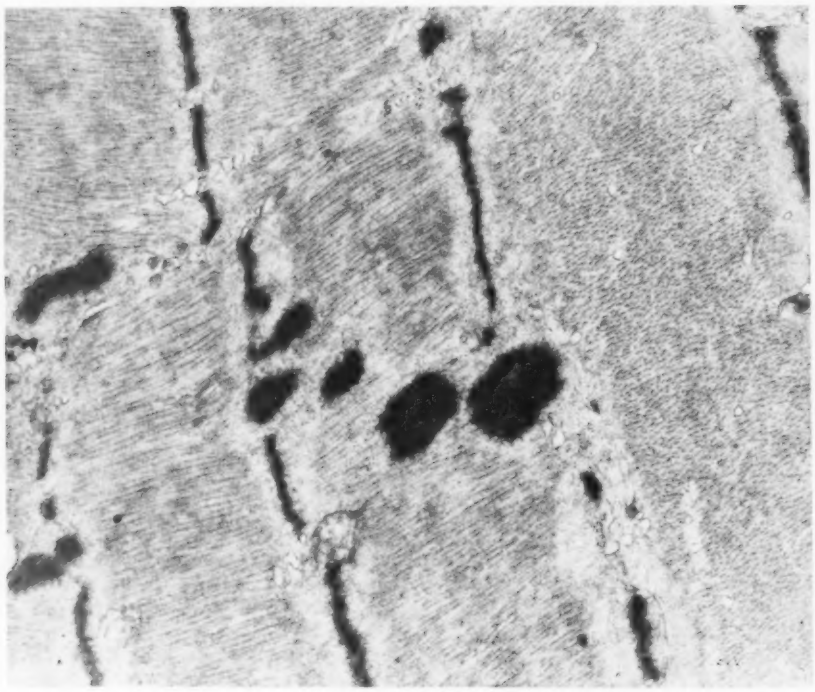

FIG. 5. A higher magnification of rod bodies, as shown in Figure 4 (× 19,000).

c) *Tubules of the T-system and of the sarcoplasmic reticulum.* In many muscle fibers the T-tubules reduplicated and ramified, thus forming transverse networks. Smaller tubules and vacuoles were closely associated with the dilated cisternae of the T-tubules (Fig. 6). Abnormal aggregates of the T-tubules and of the sarcoplasmic reticulum were encountered in 70% of the trained, 27-month-old mice (Fig. 7). The tubular aggregates occupied regions that varied in their surface area, ranging from approximately 3 × 1 μM up to 3 × 8 μM. These were mostly situated underneath the sarcolemma or near the nucleus. Tubules that were sectioned longitudinally, transversally or obliquely could be detected in the same aggregate (Fig. 7). The tubules had a diameter that ranged between 50–90 nm, but smaller and larger modules were observed. The cross-sectioned tubules revealed arrays of thin walled rings resembling honeycomb-like structures, or hexagonal formations (Figs. 7 & 8). When tubular aggregates were encountered, they then appeared in each block of the same animal and did not disappear after serial cutting of a given block. On the other hand, animals that did not reveal such tubular networks in randomized sections did indeed lack them altogether. Tubular aggregates were conspicuous in one (out of six) 19-month-old trained mice (Fig. 8).

d) *Sarcolemmal and endomysial changes.* The sarcolemma oftenly showed signs of deep invaginations thereby creating pronounced inpocketings along the fibers' periphery (Fig. 8). Concomitantly, the endomysial space revealed collagen fibrils and fibroblasts that adhered to the sarcolemma and to the basal lamina of small capillaries.

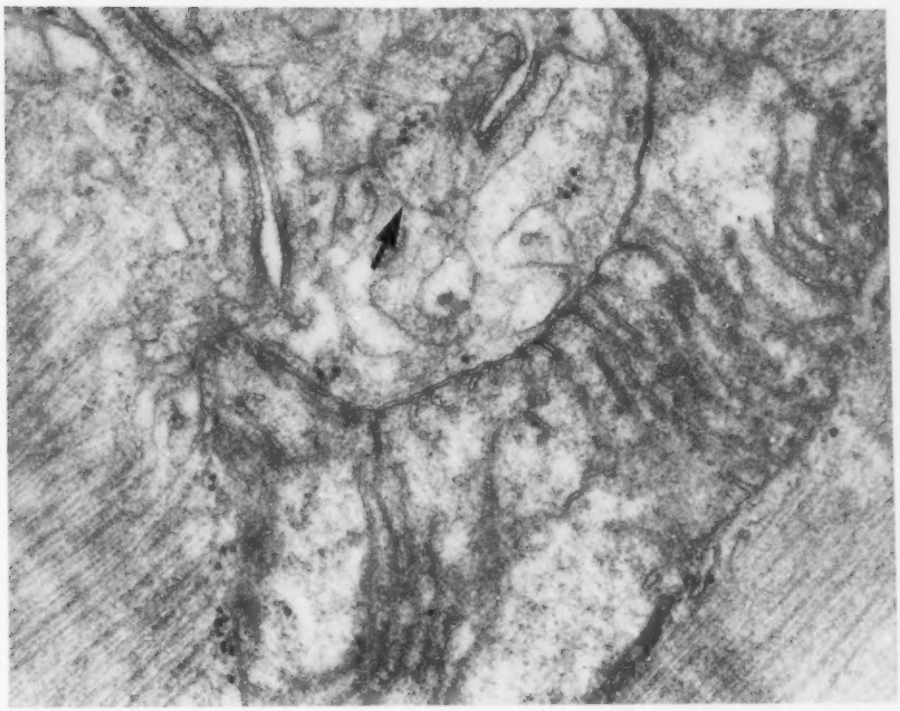

FIG. 6. A section from 27-month-old trained mouse. Note the bizarre shaped mitochondrion which has lost many of its cristae. The T-tubules and the lateral cisternae can be clearly seen (arrow) (× 56,000).

DISCUSSION

The present study has focused on the ultrastructural changes that take place in the gastrocnemius muscle secondary to enforced physical exercise at various stages of life. One of the most distinctive features characterizing the gastrocnemius muscle of aging mice is the increasing number of lipofuscin bodies and the decrease in the number of satellite cells, as previously reported by Snow (1977). Further, it became evident that the gastrocnemius muscle in aging mice undergoes atrophic changes similar to those described in skeletal muscles of aging rats (Fujisawa, 1974; 1975) and in senile humans (Tomonaga, 1977). This study points out that muscle fibers of young mice do not undergo radical changes in their internal morphology in response to endurance physical training, except for mild hypertrophy, as has been reported in healthy young individuals (McCarter, 1978; Farrar *et al.*, 1981). In contrast, senescent animals respond to endurance exercise with significant structural changes when compared with their non-trained controls. The most striking feature that is consistently observed in old trained animals relates to the T-tubular system and to a lesser extent to the tubules of the sarcoplasmic reticulum. The T-tubules oftenly underwent multiplication, thus forming tubular aggregates which are noted in 70% of all specimens obtained from old trained mice, and only in 10% of old non-trained animals. Of interest is the fact that such structures were also reported in cases of hetero-

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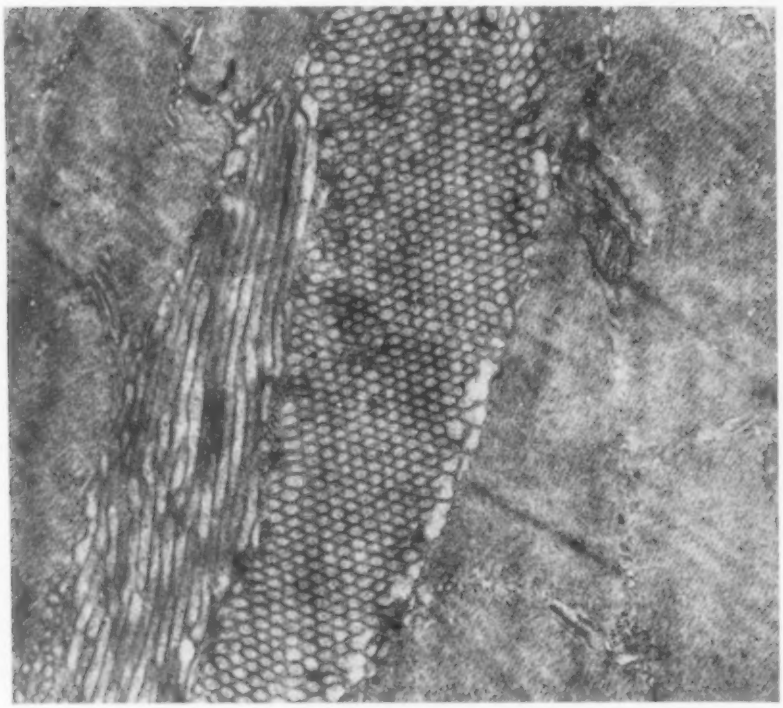

FIG. 7. A section from a muscle of an old trained mouse showing an aggregate of tubules with a honeycomb-like appearance of the transversally sectioned tubules (× 56,000).

zygous dystrophic mice (Craig and Allen, 1980), as well as in human myopathic disorders: congenital paramyotonia (Schiffer, Giordana, Monga and Mollo, 1976); hypokalemic periodic paralysis (Bergman, Afifi, Dunkle and Johns, 1970); myotonic dystrophy (Schotland, 1970); myopathy with hyperaldosteronism (Gallai, 1977); muscle weakness with normokalemia (Meyers, Gilden, Rinaldi and Hansen, 1972; Lewis, Pallis and Pearse, 1971; Morgan-Hughes, Mair and Lascelles, 1970). Multiplication and dilatation of the sarcoplasmic reticulum have also been noticed in skeletal muscles of both aged rats (Fujisawa 1975; McCarter, 1978) and humans (Shafiq, Lewis, Dimino and Schutta, 1978; Tomonaga, 1977). Recently, a stereologic study indicated a significant reduction in both the volume and surface density of the T-tubular system and that of the sarcoplasmic reticulum in the gastrocnemius of aging rats (De Coster, De Renck, Sieben and Vander Eecken, 1981). The apparent inconsistency between our recent findings and those of De Coster *et al.* (1981) could be related to the fact that most of these tubular aggregates are located subsarcolemmally, and hence, could have been perhaps overseen if examined in randomized samples. In our present study we have initially screened three grids from at least three blocks of each animal, and when no tubular aggregates were registered, additional blocks of the same animal were checked for these structures. For the most part, these tubular aggregates could be detected fairly easily, and there was no need for any ex-

FIG. 8. A section through the gastrocnemius muscle of a 19-month-old trained mouse, showing that both the sarcolemma and the adjacent nucleus (N) have undergone a pronounced invagination. Thus, a portion of the sarcoplasma containing myofilaments (F) and a tubular aggregate (T) has been caught within a nuclear pocket (× 19,000).

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tra search in order to identify them (Craig and Allen, 1980; Schotland, 1970). The pathophysiological meaning of tubular aggregates is still in the status of controversy. Some authors have expressed the belief that these structures develop as the result of the effects of various drugs (Engel, Bishop and Cunningham, 1970), or anoxia (Schiaffino, Severin, Cantini and Sartore, 1977). Duchen (1973), on the other hand, has linked their appearance to the process of denervation atrophy. One ought to agree with Morgan-Hughes *et al.* (1970) who claimed that this ultrastructural phenomenon might represent a non-specific response of skeletal muscles to as yet undetermined disturbance.

At the present stage of knowledge, we still lack sufficient data to understand the underlying factors responsible for the catabolic effects of endurance exercise upon the skeletal muscle in old animals. It might be possible that interference with the normal transmission of ionic currents through the T-system could bring about abnormal impulses which in turn could lead to an uncontrolled multiplication of tubules. Disturbances at the level of the motor end plate should be considered (Fujisawa, 1976). Tomonaga (1977) suggested that the age-related changes in human skeletal muscle could be associated with factors affecting the motor neurons.

Another issue relates to the involvement of the mitochondria in the exercise-induced changes in aging skeletal muscles. To date, there is no agreement as to the role played by the mitochondria in cell aging (Miquel *et al.*, 1980). Tonna and Severson (1971) reported on decreases in the number of mitochondria in skeletal muscles of aging mice and rats,

whereas Webb and Tribe (1974) indicated an enlargement of mitochondria in the flight muscle of an insect. The changes in the mitochondria could result from the disorganizing effects of free radicals in the membranes (Leibovitz and Siegel, 1980; Miquel *et al.*, 1980). Our findings indicate that enforced physical stress tends to enhance the regressive changes occurring in the muscular tissue of the senescent organism.

SUMMARY

The effects of endurance exercise on the gastrocnemius muscle of aging mice has been investigated by electron microscopy. Groups of 6, 19 and 27 month-old trained and non-trained control mice was used. Whereas young mice either trained or control did not show signs of muscle cell abnormalities. obvious structural changes were noted in the group of old animals. Both trained and control old mice revealed signs of variations in the size of muscle fibers including atrophy, along with myofibrillar changs in the form of focal loss of myofilaments, mitochondrial changes and an increase in the amount of endomysial collagen fibrils. In the old trained mice the most obvious abnormality consisted of multiplication of both the T-tubules and of tubules of the sarcoplasmic reticulum, thus leading to the formation of tubular aggregates in 70% of the old trained animals. It appears that in old animals endurance training tends to enhance the regressive changes that accompany the aging process of mammalian skeletal muscle.

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AGING OF THE HIGH AFFINITY GABA TRANSPORTER IN SYNAPTOSOMES FROM THE HYPOTHALAMUS OF THE RAT

D.D. WHEELER

Department of Physiology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, S.C. 29425

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INTRODUCTION

THE CENTRAL EVENT of aging appears to be the progressive loss of homeostatic regulation (von Hahn, 1970). Various homeostatic mechanisms respond more slowly and with less efficiency in the aged organism (Kohn, 1971; Gutmann and Hanzlikova, 1972; Diamond, 1978; Adelman, 1979; Masoro, Bertrand, Lepa and Yu, 1979). Since the nervous system plays a vital role in homeostasis, alterations in nervous system function are likely to be of particular significance in aging (Franks, 1970; Sohal and Sharma, 1972; Hayflick, 1976; Diamond, 1978; Lytle and Altar, 1979). The hypothalamus is probably the most important structure in the regulation of homeostasis, since all organ systems are under hypothalamic regulation to some extent. A key role of the hypothalamus in the regulation of the aging process has been proposed by Everitt (1973), who suggests that an "aging clock" which controls the rate at which the aging program is played out may be located in the hypothalamus. Evidence is accumulating that changes in the process of synaptic transmission are contributory to this decline in nervous system regulatory capacity (Kent, 1976; Pradhan, 1980).

Membrane transport processes play an integral role in the synaptic function of many transmitters. In previous studies of the transport of the putative transmitters L-glutamic acid and GABA in rat cortical synaptosomes, initial velocities of uptake were found to significantly decline when 30-month-old animals were compared to 2-month-old animals (Wheeler, 1980a; 1982). Although studies of the sodium dependence of transport showed no change in fundamental mechanism with age, changes were found in the kinetic constants which quantitate the transport mechanism. As a result, velocity of transmitter uptake at any combination of substrate and sodium concentration declined with age. Alterations with age are not common to all transport mechanisms, however; similar studies of 2-deoxy-D-glucose (2-Dg) transport showed no changes with age (Wheeler, 1981). Uptake of 2-Dg is via a low affinity, sodium independent mechanism, which is not directly related to synaptic function (Wheeler and Hollingsworth, 1979a), while uptakes of glutamate (Wheeler and Hollingsworth, 1978) and GABA (Wheeler and Hollingsworth, 1979b) are

via high affinity, sodium dependent mechanisms, which are thought to be integral to synaptic function of these compounds.

The present studies were undertaken to determine whether alterations in hypothalamic transport of GABA accompany aging. Initial velocity of uptake has been measured in aged animals (30 months) as a function of both GABA and sodium concentrations, and the results compared to previous studies of young animals. Although no change in fundamental mechanism was found to occur with age, quantitative changes were found to occur. As a result, the transport capability has declined in the aged animals.

METHODS

Male Long-Evans rats 2–3 or 30-months-old were used in this study. These rats were obtained from departmental breeding facilities and were housed two per cage under conditions of constant temperature and controlled illumination, with *ad lib.* access to food and water. The diet was obtained from Wayne Feeds (Allied Mills, Inc., Libertyville, Ill.) and contained a minimum of 24% protein, 4.5% fiber and 4% fat.

An extensive study of the suitability for membrane transport studies of the preparation and techniques used in the present study has been published (Wheeler, 1978). The methods described in this article evolved from this study. Since the details of the methods used have been published (Wheeler, 1980b), only an abbreviated outline is given below.

Preparation of synaptosomes

Following decapitation with a guillotine, the hypothalamus was rapidly dissected free, weighed, and homogenized in 50 volumes of ice cold 0.32 M sucrose. The homogenate was centrifuged for 10 min at 1085 g (2°C) to remove nuclei and cellular debris. The synaptosomes were then sedimented by centrifugation at 27,000 g for 15 min. The supernate was discarded, and the synaptosomes were resuspended in cold sucrose.

Measurement of uptake

Uptake of ^{14}C-labelled γ-aminobutyric acid (GABA) was measured by incubation of synaptosomes for one minute in a modified Krebs-Henseleit solution at 30°C (Lockwood, 1961). All incubations were carried out in a Dubnoff metabolic shaker. The concentration of GABA ([G]) in the final incubation medium was 5.0, 2.0, 1.0, 0.6667, or 0.5×10^{-6} M. Concentration of sodium ([Na]) in the incubation medium was varied by replacement of NaCl with equiosmolar Tris buffer; 0, 19, 27, 43, and 120.8 mM sodium solutions were used with each of the above GABA concentrations. Following incubation, synaptosomes were recovered on Millipore filters, washed, and the filters blotted and counted for radioactivity.

Because of the limited amount of tissue available from each animal, initial velocities were measured for only one [Na] with each of the five GABA concentrations. In order to better compare data from different animals, initial velocity for a [Na] of 120.8 mM and [G] of 0.5×10^{-6}M was also measured in each animal. This value was then utilized to normalize initial velocities. For each combination of [Na] and [G] used, initial velocity was measured in six animals in each age group.

Computer optimization

After correction for filter retention of label when incubation medium is poured through them, initial velocities were calculated, and these data were fitted to the velocity equations. A parameter optimization algorithm based on the Flexible Polyhedron approach of Nelder and Mead (1965) was used to minimize the following function:

$$J = \sum_{i=1}^{n} \left(V_{model} - V_{data} \right)^2$$

J, the objective function, is the sum of the squared errors between the velocities of uptake predicted by the equations and those observed experimentally.

RESULTS

Comparison of initial velocities

Table 1 shows a comparison of initial velocities for each [G] and [Na] used. Although there is little difference in initial velocities between the 2- and 30-month groups for the

TABLE 1. COMPARISON OF INITIAL VELOCITIES (V(30)/(2)) × 100

[G] × 10^6, M	0.5	0.6	1.0	2.0	5.0	
[Na], mM						Mean
19	87.5	86.6	109.5	94.2	101.0	95.8
27	93.6	89.2	96.0	93.0	99.6	94.3
43	96.5	90.7	100.3	82.9	88.1	91.7
120.8	78.7	76.5	78.6	66.4	70.0	74.1

One minute GABA uptake was measured in synaptosomes from 30-month old animals and compared to similar data for animals 2–3-months-old. Sodium concentration in the incubation medium was varied from 0 to 120.8 mM, while GABA concentration was varied between 0.5 and 5.0 × 10^{-6}M. For both age groups, uptake with no sodium in the incubation medium is so close to zero that the ratios are meaningless, and are therefore not included in the table.

lower [Na]'s, at 120.8mM, velocity is considerably reduced in the aged group; mean ratio of V(30)/V(2) = 0.74. This difference is significant at the 0.01 confidence level or better.

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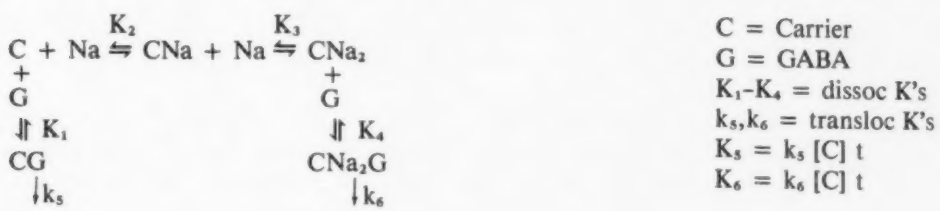
Best fit model

The 30-month data were fitted to the same model found previously to give minimal best fit to the 2-month data (Wheeler, 1980b):



$$v = \frac{\frac{K_5[G]}{K_1} + \frac{K_6[Na]^2[G]}{K_2K_3K_4}}{1 + \frac{[G]}{K_1} + \frac{[Na]}{K_2} + \frac{[Na]^2}{K_2K_3} + \frac{[Na]^2[G]}{K_2K_3K_4}} \quad (1)$$

$$v = \frac{\frac{K_2K_3K_4K_5 + K_1K_6[Na]^2}{K_2K_3K_4 + K_1[Na]^2}[G]}{\frac{K_1K_2K_3K_4 + K_1K_3K_4[Na] + K_1K_4[Na]^2}{K_2K_3K_4 + K_1[Na]^2} + [G]} = \frac{Va[G]}{Kt + [G]} \quad (2)$$

Although the scatter in the data is a little greater in the 30-month group, the model gives a good fit to the data, with an average error per data point of 4.3%, compared to 3.1% for the 2-month data. Thus it appears that aging has not resulted in a change in fundamental mechanism. However, quantitative changes in the transporter have occurred, since the best fit constants have changed with age (Table 2). Changes in K_1–K_4 result in changes in concentration of each of the several carrier species, while changes in K_5 and K_6 result in changes in maximum transport capability. The consequences of these changes in constants

TABLE 2. CONSTANTS GIVING BEST FIT

	Kn	
n	2-month	30-month
1	1.4305×10^{-5}	8.7141×10^{-5}
2	9.5948	6.9711
3	4.2442×10^{-4}	4.2347×10^{-4}
4	2.2375×10^{-6}	1.9393×10^{-6}
5	2.1944	9.9883
6	6.8278×10^{1}	4.3089×10^{1}

Initial velocity data for animals 2–3 months of age or 30 months of age were fitted to the rate equation (1) for the minimal best fit model by the computer optimization process. Shown above are the resulting constants which must be used in the rate equation to give least error between the experimental data and the corresponding values calculated from the rate equation.

$K_5 = k_5[C]_i$ and $K_6 = k_6[C]_i$; units for K_5 and K_6 are 10^{-11} mol/4 mg tissue wet weight minute.

are reduced velocities of uptake in the aged animals at certain combinations of [Na] and [G]. These changes are also reflected in alterations of the kinetic parameters which are commonly used to describe transport mechanisms, which will be described below.

Predicted velocities as a function of [Na]

The rate equation along with the best fit constants can be utilized to calculate predicted velocity as a function of [Na] for any [G] (Fig. 1). Although the shapes of the curves are similar for both ages, the curves for the 30-month animals increasingly diverge from the 2-month curves as the [Na] is increased. At a physiological [Na] of 140mM, the ratio of $(V(30)/V(2)) \times 100$ is 74.3, 73.8, 72.8, 71.0, and 68.5% at [G]'s of 0.5, $0.\bar{6}$, 1.0, 2.0, and 5.0×10^{-6}M, respectively.

$V\infty$, initial velocity of uptake at infinite [Na] and [G]

When both [Na] and [G] are infinite, the rate equation reduces to $v = K_6$. K_6 for aged animals is 43.1×10^{-11}mol/4 mg min., compared to 68.3×10^{-11}mol/4 mg min. for control animals; $V\infty(30)/V\infty(2) = 0.63$. Thus, hypothetical maximal transport capability has declined by 37% with age.

GABA concentration which gives $V\infty/2$

If only the [Na] is infinite, the rate equation reduces to

$$v = K_6[G]/(K_4 + [G]) = Jm \qquad (3)$$

When $K_4 = [G]$, $v = K_6/2$ and velocity of uptake would be half that at infinite [G]. This [G] which gives $K_6/2$ is thus a measure of the apparent affinity of the carrier for GABA under conditions of high [Na]. $K_4 = 1.94 \times 10^{-6}$ and 2.24×10^{-6} for aged and control

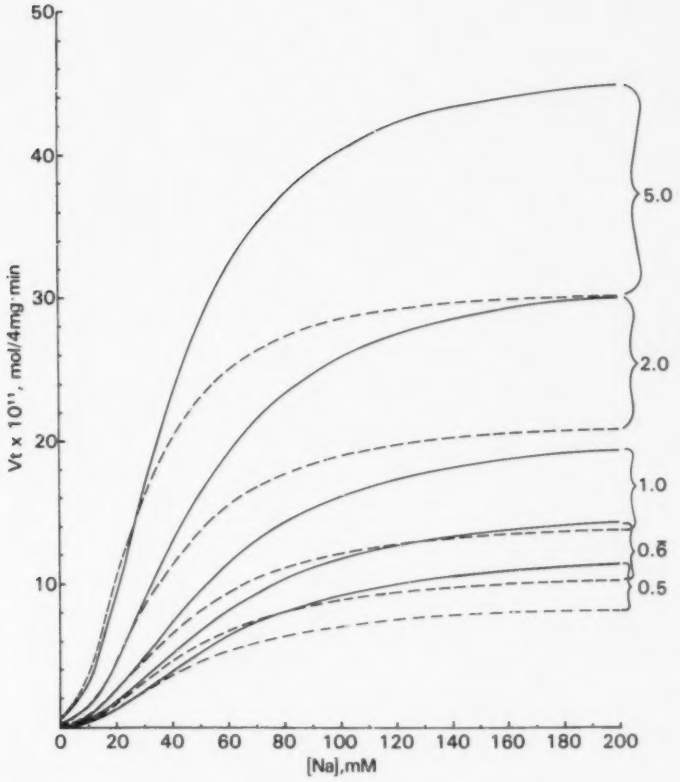


FIG. 1. Velocity of uptake as a function of sodium concentration for each of the GABA concentrations used. Equation 1 along with the appropriate best fit constants from Table 2 were used to calculate uptake for both the 2–3-month-old animals (solid lines) and the 30-month-old animals (broken lines).

animals, respectively; the ratio of $K_4(2)/K_4(30) = 1.15$. Thus the apparent affinity for GABA is some 15% greater in the aged animals.

Va, apparent maximal rate of uptake

At infinite [G], the rate equation reduces to:

$$v = \frac{K_2K_3K_4K_5 + K_1K_6[Na]^2}{K_2K_3K_4 + K_1[Na]^2} = Va \qquad (4)$$

At [Na] = 0, equation 4 reduces to $Va = K_5$, while at [Na] = ∞, $Va = K_6$. K_5 for the aged group is larger than that for the control group (Table 2). However, it should be noted that velocity of uptake at [Na] = 0 is so close to zero that there is a lot of scatter in the data, which makes the estimation of Va, (and hence Kt) of doubtful precision. At [Na] = ∞, equation 4 reduces to $Va = K_6$. As pointed out before $(K_6(30)/K_6(2)) \times 100 = 63.1\%$.

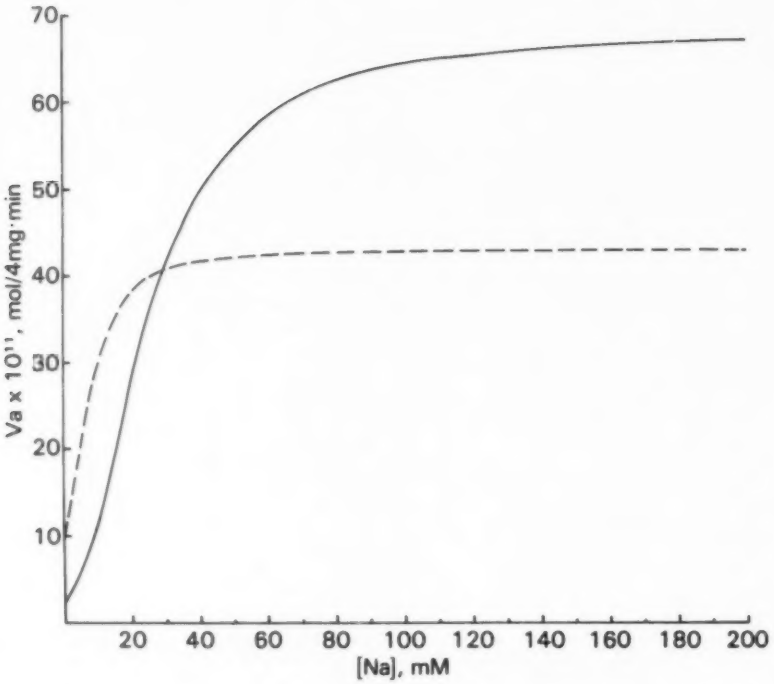


FIG. 2. Va, apparent maximal velocity of uptake, as a function of sodium concentration. Equation 4 along with the appropriate best fit constants from Table 2 were used to calculate apparent maximal velocity for animals 2–3 months of age (solid line) and animals 30 months of age (broken line).

The ratio of Va(30)/Va(2) is 1.46, 1.08, 0.82, 0.66, and 0.65 at [Na]'s of 19, 27, 43, 120.8 and 140mM, respectively (Fig. 2). Thus at 140mM [Na], maximal transport capacity has declined by 35% in the aged animals.

Kt, apparent Michaelis-Menten constant

Kt, the GABA concentration giving a rate of uptake equal to Va/2, is dependent on the [Na] and is defined by the following equation:

$$Kt = \frac{K_1K_2K_3K_4 + K_1K_3K_4[Na] + K_1K_4[Na]^2}{K_2K_3K_4 + K_1[Na]^2} \quad (5)$$

Plots of Kt as a function of [Na] are shown in Fig. 3. At low [Na]'s, Kt is larger for the aged animals than for control animals. However, the two functions cross at about 35mM [Na], so that at all [Na]'s above this value, Kt is lower for the aged animals. Since affinity is reciprocally related to Kt, the apparent affinity is greater for control animals at [Na]'s below 35mM, while it is greater for aged animals above 35mM. Thus in the physiological range of [Na], carrier affinity for GABA has increased with age. At 140mM [Na], Kt(2)/Kt(30) = 1.18; thus affinity is 18% greater for the aged animals. Optimal Kt would occur at infinite [Na], and would be given by K_4. As pointed out above, $K_4(2)/K_4(30) = 1.15$.

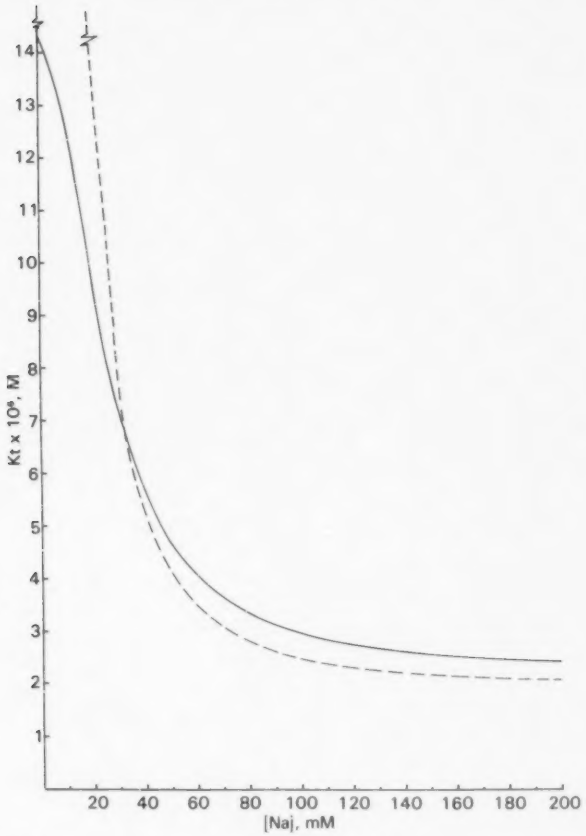


FIG. 3. Kt, the GABA concentration which gives a velocity of uptake equal to Va/2, as a function of sodium concentration. Equation 5 along with the appropriate best fit constants from Table 2 were used to calculate Kt for 2–3 months (solid line) and 30-month (broken line) animals.

For control animals Kt([Na] = 140mM)/K_4 = 1.17; for aged animals, Kt([Na] = 140)/K_4 = 1.15. Thus for both groups, at physiological [Na], the carriers are functioning close to their optimal affinities for GABA.

Jm, rate of uptake at infinite [Na] and a given [G]

Jm is defined by equation 3:

$$Jm = \frac{K_6[G]}{K_4 + [G]}$$

At all [G]'s used, Jm has declined with age. The ratio of Jm(30)/ Jm(2) varies from 0.71 for 0.5 × 10^{-6}M [G] to 0.66 for 5 × 10^{-6}M (Fig. 4). As [G] approaches infinity, Jm approaches K_6 as a limit; (Jm(30)/ Jm(2)) × 100 = 63.1%. Thus the theoretical transport capability under conditions of high [Na] has declined substantially with age.

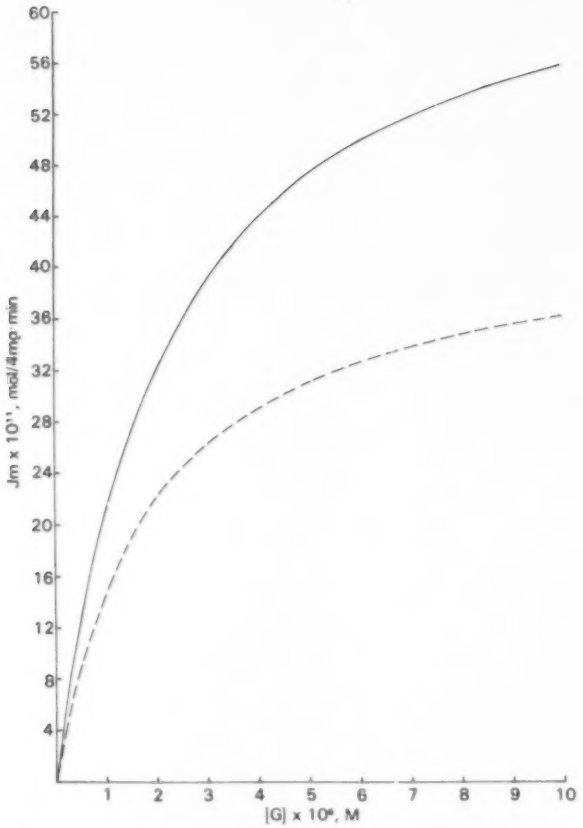


FIG. 4. Jm, rate of uptake at infinite [Na], as a function of GABA concentration. Equation 3 along with the appropriate best fit constants from Table 2 were used to calculate Jm. Solid line, 2 month; broken line, 30 month.

K_{Na}, the [Na] giving a velocity of uptake equal to Jm/2

Kt is a measure of the apparent carrier affinity for GABA; analogously, K_{Na} is a measure of the apparent carrier affinity for sodium. K_{Na} can be found graphically from Fig. 1, or K_{Na} can be found by solving the rate equation for [Na] when the velocity equals Jm/2. The following quadratic equation results:

$$(K_1K_4 + K_1[G])[Na]^2 - K_1K_3K_4[Na] + (K_2K_3K_4)((2K_5/K_6) - 1)[G] + (K_2K_3K_4)((2K_4K_5/K_6) - K_1) = 0 \quad (6)$$

Figure 5 shows a plot of K_{Na} as a function of $-\log$ [G]. Although the shapes of the curves are similar, K_{Na} is lower for aged animals at all [G]'s. The ratios of $K_{Na}(30)/K_{Na}(2)$ are 0.84, 0.84, 0.83, 0.82, and 0.81 at [G]s of 0.5, $0.\overline{6}$, 1.0, 2.0, and 5.0 $\times$ 10^{-6}M, respectively. Thus aging has resulted in an increase in the apparent affinity for [Na], since affinity is reciprocally related to K_{Na}.

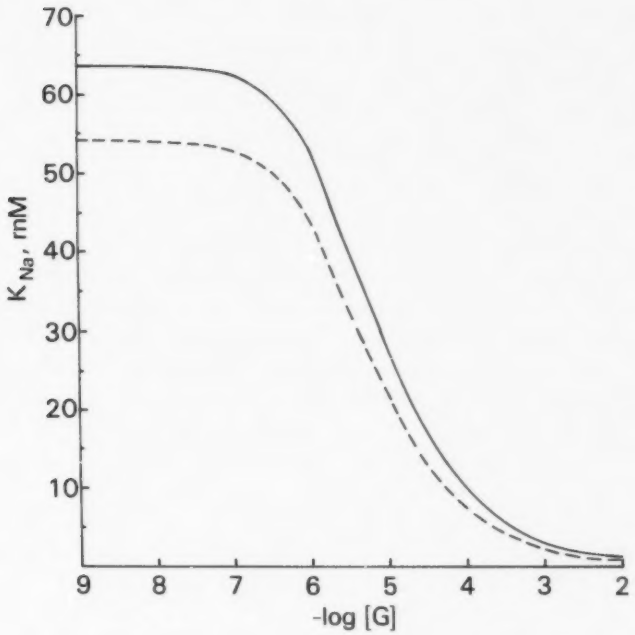


FIG. 5. K_{Na}, the sodium concentration giving a velocity of uptake equal to Jm/2, as a function of GABA concentration. Equation 6 along with the appropriate constants from Table 2 were used to calculate K_{Na}. Solid line, 2 month; broken line, 30-month.

TABLE 3. PERCENTAGE OF TOTAL UPTAKE AS CNa_2G

[Na], mM	2-month	30-month
10	83.0	86.8
20	95.1	96.3
30	97.8	98.3
40	98.7	99.1
50	99.2	99.4
60	99.4	99.6
70	99.6	99.7
80	99.7	99.8
90	99.7	99.8
100	99.8	99.8
110	99.8	99.9
120	99.9	99.9
130	99.9	99.9
140	99.9	99.9

The rate equation for the best fit model (equation 1) was used along with the best fit constants from Table 2 to compute uptake by each of the two translocation pathways (as CNaG and as CNa_2G) for 2-3-month-old animals and for 30-month-old animals.

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Contribution of each pathway to total uptake

Although the model allows uptake to take place as CG, most of the uptakes takes place as CNa_2G in both age groups (Table 3). For both control and aged animals, more than 95% of total uptake is via CNa_2G when the [Na] is 20mM or above. Thus, for both ages, only at extremely low [Na]'s does uptake as CG contribute measurably to uptake.

DISCUSSION

The results confirm the hypothesis that alterations in the hypothalamic GABA transporter accompany aging. In the physiological range of [Na], initial velocity of uptake declined by about 25% in the 30-month age group. Although the fundamental mechanism by which sodium and GABA interact with carrier apparently does not change with age, the constants which quantitate the model were found to change. Those kinetic parameters related to maximal transport capability declined with age, while apparent carrier affinities increased with age. K_6, maximal transport capacity under conditions of high [Na] and [G], declined by 37% in the aged group. Since $K_6 = k_6[C]_t$, either the translocation constant (k_6) or the total carrier concentration, or both, must have declined with age. Similar inferences can be made from the observed decline in Va and Jm. K_4, which is reciprocally related to the apparent carrier affinity for GABA under conditions of high [Na], declined by 15% with age, thus indicating an increase in apparent carrier affinity. The observed reductions in Kt (except at low [Na]), are also indicative of an increased affinity for GABA. Apparent carrier affinity for sodium also increased with age, since K_{Na} was found to be lower in the aged group for all [G]'s used.

The observed alterations in kinetic constants in the aged group of animals is likely to have important physiological consequences. The decline with age in apparent maximal velocity in the physiological range of sodium concentration is likely to be reflected in a decline in GABA concentrative capacity of the presynaptic nerve endings. Thus less GABA would be available for release during depolarization of the nerve terminal, and hence the postsynaptic effect of released transmitter might be delayed and of lesser magnitude. The reduced rate of GABA uptake might also be reflected in a prolongation of postsynaptic effects. Thus the postsynaptic effects of the release of GABA might be expected to be less in magnitude and of longer duration in aged animals compared to young animals.

One of the most significant aspects of aging is the decline in regulatory ability in the face of stress. Perhaps no other CNS structure plays so vital a role in this homeostatic regulation as does the hypothalamus. Thus a decline in the ability of hypothalamic presynaptic endings to remove the transmitter GABA, which has been demonstrated in the present study, might be expected to have widespread deleterious effects on synaptic transmission, thereby contributing to the observed decline in regulatory ability which is such a common feature of aging.

Four studies of the aging of synaptosomal transport mechanisms have now been completed in this laboratory. In a study of the glucose transporter, no changes were found when initial velocities of 2-Deoxy-D-glucose were compared in cortical synaptosomes from 2- and 30-month animals (Wheeler, 1981). This glucose transport mechanism, which is common to all nerve cells, is a low affinity, sodium independent mechanism which functions in maintaining cellular glucose levels essential to the metabolic role of glucose. In addition to the present study, two other studies of the aging of synaptosomal transport of putative transmitters have been made. The results for both GABA (Wheeler, 1982) and

glutamic acid (Wheeler, 1980a) in cortical synaptosomes are quite similar to those from the present study. Thus these transport mechanisms, which are high affinity, sodium dependent mechanisms integral to a synaptic role for these compounds, are more labile to the deleterious effects of age.

SUMMARY

Initial velocity of GABA uptake in hypothalamic synaptosomes has been measured in aged animals as a function of both sodium and GABA concentration and compared to previous studies in young animals. The data give good fit to the model found previously to give minimal best fit to control data. Thus it appears that aging has not resulted in a change in fundamental mechanism by which carrier, sodium, and GABA interact in the process of transport. However, changes were found in the constants which quantitate the model. As a consequence, there is a reduction in transport capability in the aged animals which is particularly apparent at the higher sodium concentrations.

The best fit constants were used along with the rate equation from the model to calculate several kinetic parameters which are useful in comparing transport mechanisms. In the physiological range of sodium concentration, those kinetic parameters related to maximal transport capabilities declined with age, while apparent carrier affinities increased with age. Similar results were found in previous studies of GABA (Wheeler, 1982) and glutamic acid (Wheeler, 1980a) transport in cortical synaptosomes.

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BRIEF ANNOTATION

AN INVERSE RELATIONSHIP BETWEEN MAMMALIAN LIFESPAN AND CARTILAGE CELLULARITY

C.H. EVANS

Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine,
986 Scaife Hall, Pittsburgh, PA 15213

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GERONTOLOGICAL STUDIES have revealed a number of interesting correlations between species lifespan and particular anatomical and physiological characteristics. With mammals, for example, longevity increases with increasing body weight and brain weight (Sacher, 1959), but decreases with increasing rates of heart-beat and breath-time (Gould, 1977). Although such data do not necessarily help to explain the different specific lifespans, it is to be hoped that any theory which does so, will account for these correlates.

In this communication, I would like to draw attention to a striking negative relationship between the logarithm of the lifespans of a number of different mammalian species, and the density of chondrocytes in the articular cartilage of their femoral condyles (Fig. 1). The data for the number of chondrocytes per unit volume of cartilage are those of Stockwell (1971) and the different mammalian lifespans are taken from Comfort (1964).

It is interesting that the data points fall onto two, approximately parallel, straight lines. The reason for the negative correlation shown in Fig. 1 is not apparent. Stockwell (1971) drew attention to the roughly inverse correlation between cartilage cellularity and body weight; as just noted, body weight is positively correlated with lifespan. Differences in cellularity of the articular cartilage cannot be explained on the basis of providing a reservoir of functional cells to replace "worn-out" cells and thus maintain operational integrity during extended lifespans, as the cartilages of the longer living species are the less cellular. Stockwell's data also show an inverse relationship between cellularity and cartilage thickness. Thus all the cartilages studied had about the same number of chondrocytes beneath 1mm^2 of articular surface.

Articular chondrocytes are faculatively non-mitotic cells which undergo cellular aging *in vivo* and *in vitro* (Evans and Georgescu, 1983). Furthermore, we have suggested that their cellular senescence engenders primary osteoarthritis, an age-related disease.

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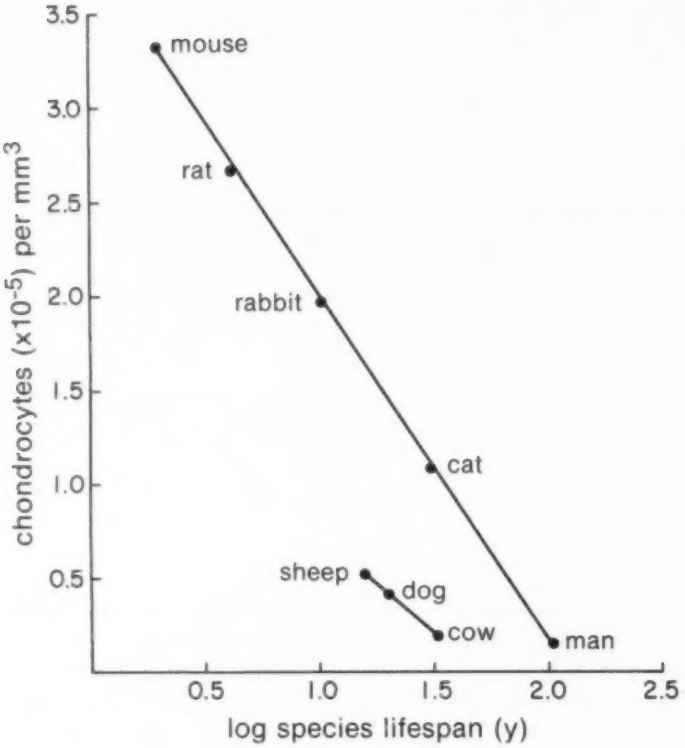

FIG. 1. Relationship between cartilage cellularity and $\log_{10}$ lifespan for various mammals.

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AGE-RELATED ULTRASTRUCTURAL CHANGES IN HUMAN EMBRYONIC LUNG FIBROBLASTS

A.M. BOAK,* A.H. BITTLES,* and P.J. QUINN†

Department of Human Biology* and Biochemistry,† Chelsea College,
University of London, London SW3 6LX, U.K.

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Abstract—Scanning and transmission electron microscopy were used to examine human embryonic lung fibroblasts at different population doubling levels. Scanning electron microscopy of cells at population doubling levels 26, 45 and 59 did not reveal a significant change in cell size with increasing age. However, transmission electron microscopy of cells at population doubling levels 19 and 45 showed an increase in nuclear lobes, a decrease in the number of ribosomes associated with rough endoplasmic reticulum, and changes to the internal structure of mitochondria on increasing population doubling level. No other previously reported age-related changes were found.

INTRODUCTION

THREE PHASES of fibroblast growth in tissue culture can be recognised on the basis of population doubling level (Hayflick, 1965). Changes in cell morphology and in the shape and arrangement of internal organelles during the ageing process have been observed with scanning and transmission electron microscopy. An increase in the surface area of late passage fibroblasts compared with early passage cells, for example, has been reported by Maciera-Coelho (1973) and Basler *et al.* (1979). Early transmission electron microscopic studies revealed that the nuclei became more lobed as fibroblasts aged in tissue culture (Robbins *et al.*, 1970; Brock and Hay, 1971; Lipetz and Cristofalo, 1972); the nuclei of early passage cells were almost invariably ovoid in shape with few if any indentations. Subsequently, Johnson (1979) reported that some early passage cells of the human embryonic lung fibroblast line IMR90 had multilobed nuclei and that older cells had chromatin condensed along the nuclear envelope.

Apart from the nucleus, various studies have suggested changes in the number and/or distribution of subcellular organelles during cell ageing. The accumulation of lysosomes (Brock and Hay, 1971) and an increase in lysosomal enzyme activity (Milisauskas and Rose, 1973; Cristofalo and Kabakjian, 1975) is often observed. Some workers have reported an age-related increase in the number of Golgi bodies (Lipetz and Cristofalo, 1972; Basler *et al.*, 1979) whereas others found no significant change in these organelles (Robert, 1977). Similarly, a report that the cysternal space of the endoplasmic reticulum was dilated in young cells (Lipetz and Cristofalo, 1972) has not been confirmed by later studies (Basler *et al.*, 1979; Johnson, 1979), which showed rather that the endoplasmic reticulum

tended to be dilated in older cells. There was, however, general agreement that the number of membrane-associated ribosomes decreased with age. In other cell lines, such as chick embryo fibroblasts, some workers report an increase in the number of mitochondria with cell age (Brock and Hay, 1971) while others observe no significant change (Lipetz and Cristofalo, 1972); changes in mitochondrial morphology are also disputed. Mitochondria of IMR90 cells have been classified by Johnson (1979) into Type I, which are long and condensed, and Type II, which are short and rounded; Type I mitochondria were reported to predominate in old cells.

The present study was undertaken to examine age-related morphological changes, in a cell line of human embryonic lung fibroblasts not previously studied, to help resolve some of the differences reported in subcellular organelles associated with senescence. The study was based both on scanning and transmission electron microscopy of cells that had undergone specific numbers of population doublings.

MATERIALS AND METHODS

Human embryonic lung fibroblasts (Lot number 1044) were obtained from Gibco Europe Limited, Paisley, Scotland, as were all other materials required for cell culture. Plastic petri dishes were purchased from Sterilin Limited, Teddington, England, and solutions required for scanning and transmission electron microscopy were from Polaron Equipment Limited, Watford, England. The fibroblasts were grown on Nunc plastic culture flasks using Glasgow Modification of Eagle's Medium supplemented with 10% fetal calf serum, Penicillin (100 units ml^{-1}) and Streptomycin (100 $\mu g\ ml^{-1}$). For scanning and transmission electron microscopy cells at population doubling levels 19, 26, 45 and 59 were plated onto plastic petri dishes (30 mm in diameter) containing glass coverslips. The maximum population doubling level obtained was between 55 and 61. The cultures were routinely checked for mycoplasma contamination by fluorescent microscopy (Chen, 1977).

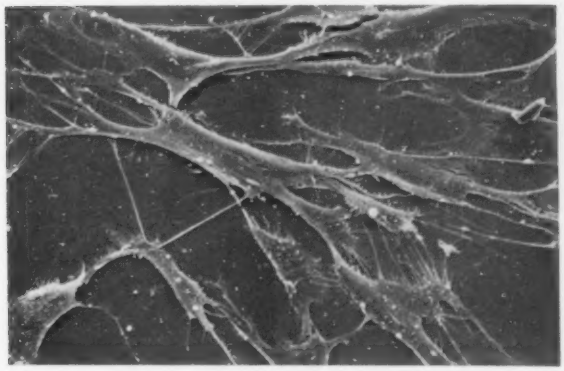
The fibroblasts were fixed for both scanning and transmission electron microscopy with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4, containing 0.1M sucrose, for two hours at 4°C. Cells for transmission electron microscopy were post-fixed for one hour at 4°C in 1% osmium tetroxide in the same sodium cacodylate buffer, removed from the coverslips using a silicone 'policeman' and embedded in 2% (w/v) agar. Dehydration for both procedures was in a graded ethanol series (50 to 100% v/v) and all subsequent preparation of samples employed standard procedures.

RESULTS

The scanning electron micrographs in Fig. 1 show an increase in the mean area of growth surface covered by individual cells as the population doubling level increases. The mean dimensions of the cells at population doubling level 26 were 90 μ by 19 μ whereas at population doubling levels 45 and 59 the dimensions were 78 μ by 25 μ and 104 μ by 28 μ, respectively. This represented a 1.6-fold increase in the area covered by individual cells between population doublings 26 and 59.

The structure and organization of subcellular organelles were examined in human embryonic lung fibroblasts after 19 and 45 population doublings by transmission electron microscopy. Figure 2 illustrates morphological changes associated with the nuclei of these cells. Old cells have nuclei that vary considerably in shape and most characteristically are multilobed. Younger cells, on the other hand, tend to have smooth nuclear envelopes.

Changes in the endoplasmic reticulum and Golgi apparatus were also observed between young and old cells. Although no quantitative assessment of the proportions of rough-surface to smooth-surface endoplasmic reticulum was made there were obvious differences in staining density between the two experimental groups. Figure 3, for example, shows regions of the cell containing rough-surface endoplasmic reticulum. The stain density is much greater in the young compared with the old cells in preparations that were



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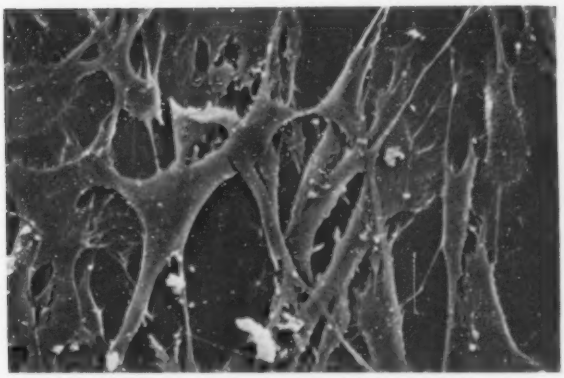

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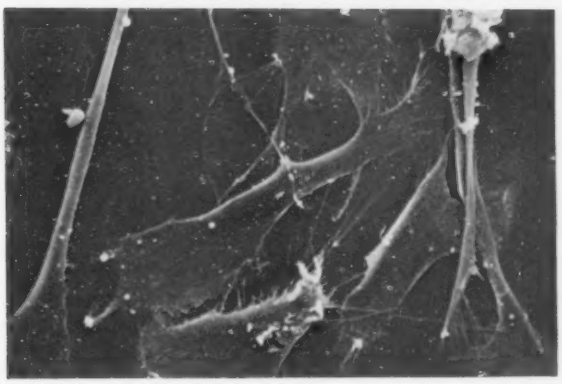

c

FIG. 1. Scanning electron micrographs of human embryonic lung fibroblasts after (a) 26, (b) 45 and (c) 59 population doublings. Cells were subcultured for 24 hours at 37°C before fixing and shadowing (magnification × 400).

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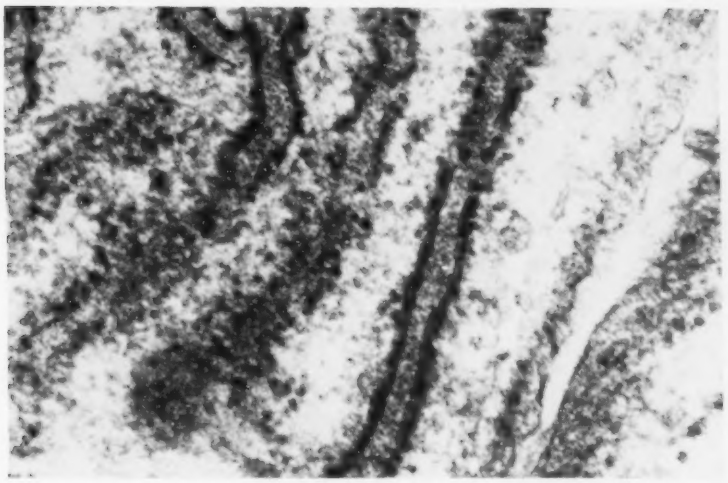
a

b

FIG. 2. Electron micrographs of thin sections of human embryonic lung fibroblasts after (a) 19 and (b) 45 population doublings showing the structure of the nuclei (magnification × 13300).

treated identically for examination. One interpretation is that the endoplasmic reticulum of young cells contains a higher density of attached ribosomes. There did not appear to be any difference in the size of the cysternal space and this was true in general also for the cysternal space of smooth endoplasmic reticulum. Prominent Golgi structures were invariably found in cells that had undergone 19 population doublings. An example of a



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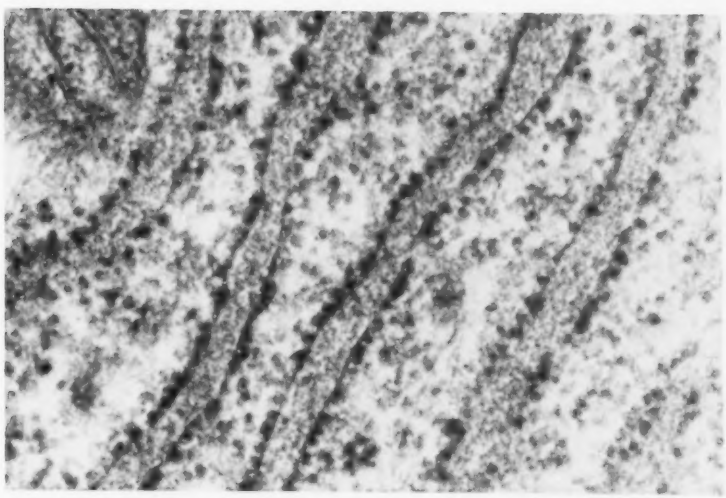

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FIG. 3. Electron micrographs of thin sections of human embryonic lung fibroblasts after (a) 19 and (b) 45 population doublings. Regions of the cells containing rough-surface endoplasmic reticulum are shown (magnification × 118000).

Golgi complex in such fibroblasts is shown in Fig. 4. In contrast, cells that had undergone 45 population doublings had no recognisable Golgi bodies.

Differences in staining density were observed also between mitochondria from young and old cells. Thus the mitochondria of young cells appeared to be more densely stained and to have better developed cristae membranes than their older counterparts. Although

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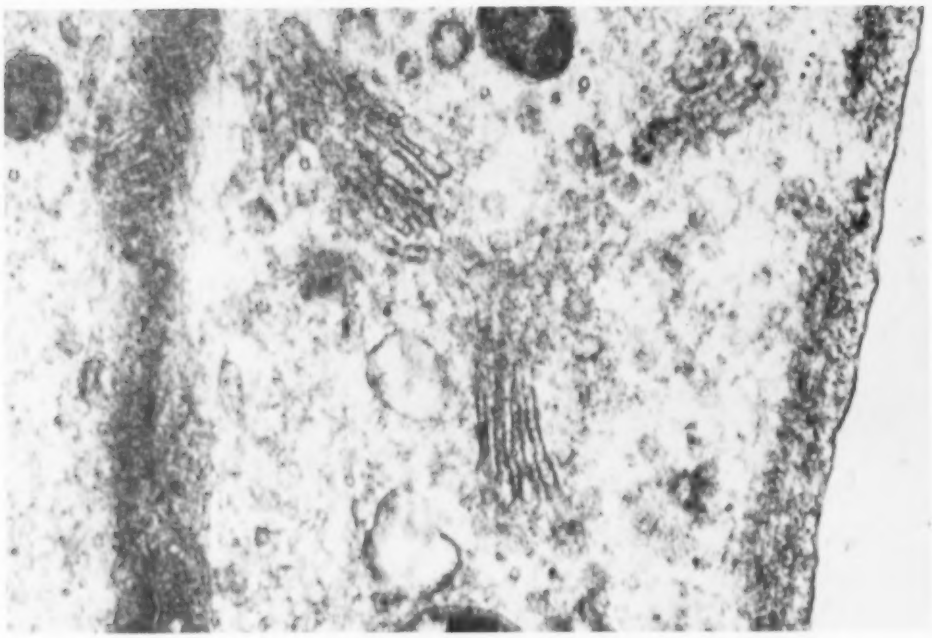


FIG. 4. Electron micrograph of a thin section of human embryonic lung fibroblasts after 19 population doublings showing a region of the cell containing Golgi membranes. Small vesicles can be seen budding off the ends of the flattened stacks of Golgi membranes (magnification × 86000).

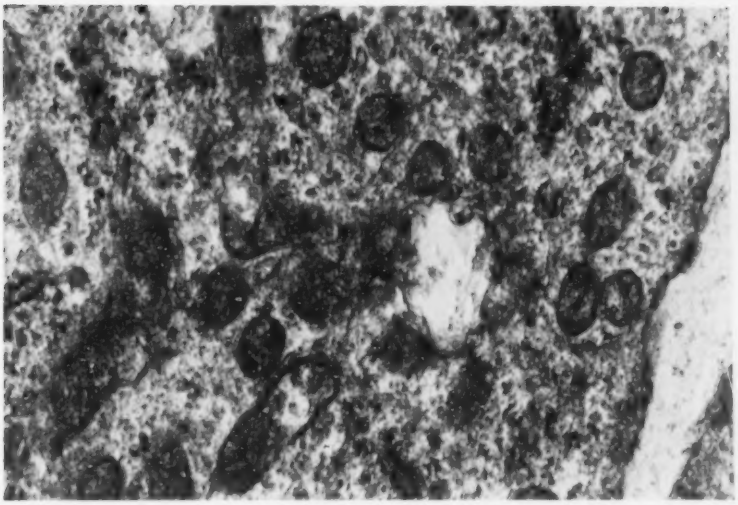
differences in morphology are apparent in Fig. 5 which illustrates the contrast in staining density, no consistent morphology could be ascribed to ageing in these cells. Thus elongated, round and L-shaped mitochondria were observed at about the same frequency in young and old cells. No significant changes in the numbers or types of lysosomes were detected between cells that had undergone 19 or 45 population doublings.

DISCUSSION

Some of the features we have observed during the ageing of human embryonic lung fibroblasts in culture are consistent with earlier reports but others are to the contrary.

Although most of the old cells appeared to have ovoid nuclei, a number exhibited a multi-lobed structure similar to that described by Robbins *et al.* (1970). Unlike the report of Johnson (1979), however, we did not see a significant difference in the amount of condensed chromatin associated with nuclear membranes on ageing, which is consistent with reports by other authors (Lipetz and Cristofalo, 1972; Basler *et al.*, 1979).

Our studies have not shown any consistent changes in the dimensions of the cysternal space of the endoplasmic reticulum between young and old cells. This is in contrast both to the observations of Lipetz and Cristofalo (1972) who found a decrease in the diameter of the cysternal space with ageing, and to other workers who reported the opposite effect (Basler *et al.*, 1979; Johnson, 1979). An accurate determination of the dimensions of the space is not easy because the precise location of the membrane is often obscured by



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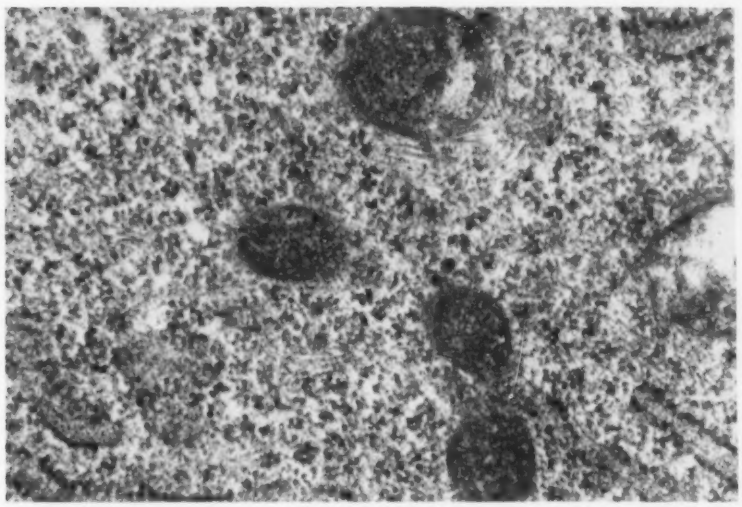

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FIG. 5. Electron micrographs of thin sections of human embryonic lung fibroblasts after (a) 19 and (b) 45 population doublings illustrating the morphology of the mitochondria (magnification × 56000).

membrane-associated ribosomes. In addition, we observed that an increase in cysternal space could occur during glutaraldehyde treatment, part of the fixation process, after detachment of the cells from the substrate; by comparison cells fixed prior to removal from the coverslips showed no apparent change. One consistent feature, however, is that the number of attached ribosomes decreased with cell age. Some workers have correlated

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distended endoplasmic reticulum in old cells with large and active Golgi bodies and an increase in protein turnover (Basler *et al.*, 1979). We have not observed any prominent Golgi bodies in our fibroblasts after more than 40 population doublings.

The young cells studied appeared to have more mitochondria than the older cells but a more comprehensive study of serial sections would be required to confirm this observation. Both types of mitochondria recorded by Johnson (1979) were seen in young and old cells alike. The presence of the two mitochondrial types, however, could result from sectioning mitochondria through different orientations within the cells. The mitochondria of the young cells stained more densely than did those of old cells. Possibly this was because the cristae of the young cells were more intact than in the old cells, as earlier suggested by Lipetz and Cristofalo (1972). None of the U-shaped or T-shaped mitochondria observed by Brock and Hay (1971) or Johnson (1979) were found in either the old or the young cells, although a number of the mitochondria were bent (L-shaped).

The results of all previous studies showed an accumulation of lysosomes in old cells. In the present study both young and old cells showed a large number of densely staining lysosomes and also several large, translucent vacuoles which were possibly lipidic in nature (Lipetz and Cristofalo, 1972; Johnson, 1979). No significant difference was found in the numbers of lysosomes between young and old cells.

The old cells used in the present study showed some, but by no means all, of the age-related changes reported by previous authors. These old cells were at population doubling level 45, whereas the maximum population doubling level found for this cell line was 55 to 61. It is possible that some of the changes associated with senescence do not occur until late in Phase III and thus are not found in the cells of the 45th population doubling level used in this study.

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RESOURCE AVAILABILITY, MATERNAL EFFECTS, AND LONGEVITY

MICHAEL LYNCH AND RICHARD ENNIS

Dept. of Ecology, Ethology, and Evolution, 101 Vivarium, University of Illinois, Champaign, IL 61820

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Abstract—Experiments with a clone of the cladoceran *Daphnia pulex* indicate that the nutritional conditions of the maternal environment play a major role in determining the progeny's phenotype. Apparently, by influencing the physiology and/or morphology of individuals during early development, maternal investment not only enhances juvenile survival but has long-lasting, favorable effects on the progeny's ability to convert resources into growth and reproduction as well as negative repercussions for adult survival of the progeny. Life-span may also be radically altered by modifying food schedules within an individual's life. Neither reproductive effort nor rate of living hypotheses can explain longevity variation within *Daphnia* clones; rather the onset of senescence appears to be associated with a general breakdown in the ability to incorporate energy into biomass. Analysis of our results as well as earlier data with a "rate of aging-threshold vitality" model suggests that increasing the availability of food to an individual increases the rate of aging while decreasing the threshold vitality necessary for survival and that increasing maternal investment increases both the vitality at birth and the rate of aging of the progeny.

INTRODUCTION

PARENTAL EFFECTS, especially maternal effects (i.e., the influence of the maternal environment on the phenotypic expression of progeny), are well-documented in a diversity of animals (David, 1961; Falconer, 1981) and have recently attracted a great deal of attention from experimental gerontologists (Lints, 1978). However, with the notable exception of David's (1961) thorough investigation with *Drosophila*, almost all existing work on parental effects has focused on maternal age effects, i.e., the influence of maternal age on offspring quality, and the interpretation of many of these studies is open to criticism (Lints, 1978). Except for Robertson and Salt, 1981, very little consideration has been given to the progeny character that would seem most relevant to gerontologists—longevity.

Yet since a diversity of characters including egg size (David, 1962) and nucleic acid content (Tsien and Wattiaux, 1971), developmental rate (Delcour, 1969), body size (Falconer, 1981), and genotype (Bridges, 1929; Valentin, 1973; Kram and Schneider, 1978) are known to be influenced by maternal effects, and since these traits are often correlated with longevity (Comfort, 1979; Lamb, 1977; Lints, 1978), it is not unreasonable to expect early maternal influences (operating solely through the egg) to be important determinants of an individual's life-span. The demonstration of Lansing effects, cumulative and reversible parental age effects, on the life-span of a diversity of organisms (Lansing, 1947;

Ashby and Wangermann, 1954; Murphy and Davidoff, 1972) is further supportive of such an expectation.

We have investigated this problem at an unparalleled level of simplicity using a single clone of the parthenogenetic cladoceran, *Daphnia pulex*. In the following pages we demonstrate not only that the nutritional environment of the mother has multiple and long-lasting effects on the phenotypes of her progeny, but also that these effects are highly dependent on the environment of her offspring. In addition, we consider our results in light of recent speculations on the mechanisms of aging and examine a general model that may be of utility in explaining life-span variation among individuals under controlled environmental settings.

METHODS

All *Daphnia* used in this experiment were third generation descendents of a single parthenogenetic female drawn from a laboratory stock of an obligately unisexual strain of the Group A composite genotype (Lynch, 1983); as parthenogenesis in *Daphnia* is of the ameiotic type, all experimental individuals were genetically identical, barring mutations. The progeny from the first clutch of the stem mother (the grandparental generation) were separated into high and low food treatments until they released their first clutch, the members of which (the parental generation) were treated in an identical manner as their parents (Fig. 1). These two preliminary generations insured that all experimental individuals within lines would have identical past histories.

The first clutch progeny of the parental generation on high food were randomly divided into high (HH) and low (HL) food lines; similarly, progeny from low food lines were separated into high (LH) and low (LL) food lines. Thirty individuals were exposed to each treatment. In the absence of maternal effects the HH and LH lines should be identical to each other, as should the HL and LL lines.

The food supply consisted of a 5:1 cell density ratio of pure cultures of *Scenedesmus dimorphus* and *Chlamydomonas reinhardti*: high food - 80,000 and 16,000 cells/ml, and low food - 8,000 and 1,600 cells/ml, respectively. The algae were grown on a medium modified from Guillard and Lorenzen, 1972 and supplemented with vitamins; new cultures were inoculated every 7 days, insuring that algae used as food always came from cultures aged 1–2 weeks. Cell densities were determined with a hemacytometer, centrifuged from the algal medium, and then diluted in a defined zooplankton medium (modified from Murphy, 1970) to the appropriate density.

Algae and *Daphnia* were grown in a Percival incubator at 20°C on a 12:12 hr light:dark cycle. *Daphnia* were maintained individually in 40 ml of medium, with fresh medium being substituted every other day. Every in-

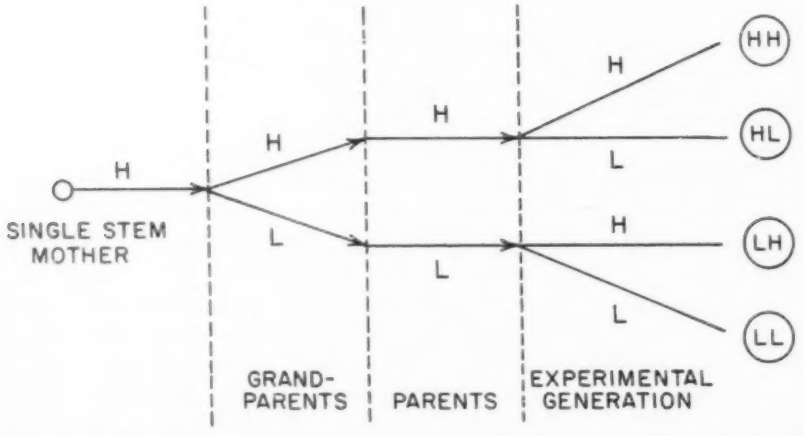

FIG. 1. The experimental design. Three preliminary generations were completed prior to the experimental generation. H and L refer to high and low food levels. The four experimental lines are HH, HL, LH, and LL, the first letter referring to the conditions under which the mother was raised, the second to the conditions under which the measured individual was raised.

dividual was examined daily for survival and measured for growth (total length) and reproduction (size of clutch carried, and/or number and size of new offspring released) under a Wild M-8 dissecting microscope.

The relative amounts of resources allocated to growth and reproduction by the different lines were estimated from dry weight measures. Since eggs and somatic tissues in cladocerans are approximately calorically equivalent on a dry weight basis (Schindler, 1968; Snow 1972), proportional investments calculated in terms of dry weight should be very similar to ratios based on calories.

The amount of dry weight invested in growth (μg/day) was derived by converting changes in length between instars to dry weight by the length-weight regression appropriate to the food supply and dividing by the instar duration. The length-weight relation was determined by drying individuals of various lengths for 48 hrs at 60°C and then weighing them to the nearest microgram on a Mettler M22 electrobalance. Of the adult females weighed, only those that had recently emptied their ovaries by producing a clutch were utilized, the clutch being removed prior to weighing; this insures that estimated rates of investment in growth are not biased by changes in ovary or progeny weights. Logarithmic regressions of dry weight (μg) on length (mm) were highly significant ($p < 0.0001$): $W = 11.04L^{2.05}$ for individuals grown on high food, and $W = 7.81L^{2.16}$ for individuals grown on low food.

The amount of dry weight invested in reproduction (μg/day) was estimated as (number of eggs produced/instar $\times$ dry weight/egg $\div$ instar duration). Mean dry weights for freshly deposited eggs are 2.11 and 1.73 μg under high and low food conditions.

RESULTS

The growth, survivorship, and reproductive schedules for the four cohorts are illustrated in Fig. 2. Significant maternal effects with respect to growth existed for the individuals grown on low food to those individuals whose mothers were grown on high food (HL) consistently having larger sizes than the LL control lines. Although the effects are not as pronounced, the maternal environment also influenced the growth of the high food lines. As the growth curves within food treatments are essentially parallel after 10 days, most of the maternal influence on growth must have been expressed early in life.

The maternal environment had no influence on the reproductive schedule of the high food lines, but there were pronounced differences between the low food lines. Until about the twelfth day of life (through the second clutch), the HL individuals carried clutches that were substantially larger than those of LL females and nearly identical to those of the high food lines; immediately thereafter, the low food lines converged and remained similar with respect to reproduction. The important point is that early in life HL individuals invest nearly as much in reproduction (on an absolute scale) as would be the case if they were growing on high food.

Thus, maternal investment has a major influence on the progeny's ability to harvest energy at relatively low food concentrations. When the HL and LL lines are compared, it becomes clear that a 0.38 μg dry wt. (22%) additional investment per egg is converted into an $\sim$100% increase in the progeny's ability to incorporate energy into growth and reproduction (F) early in life (Fig. 3). Such an increase in net energy intake implies that maternal effects alter the physiology and/or morphology of individuals during development in ways that generate very major phenotypic changes later in life.

Two differences of importance with respect to survivorship also arose between the lines (Fig. 2, Table 1). Early survivorship was significantly lower for the LL than for the other lines. Being raised on high food or descending from a mother raised on high food nearly guaranteed survival to maturity. However, individuals grown on low food but derived from high food mothers (HL) experienced a very significant and precipitous increase in mortality after 40 days; no HL individual lived beyond 50 days. The three remaining lines exhibited very similar l_x schedules after 50 days, with $\sim$30% of the individuals surviving to 50 days and several surviving as long as 70 days.

One characteristic for which the lines were remarkably constant was the proportion of

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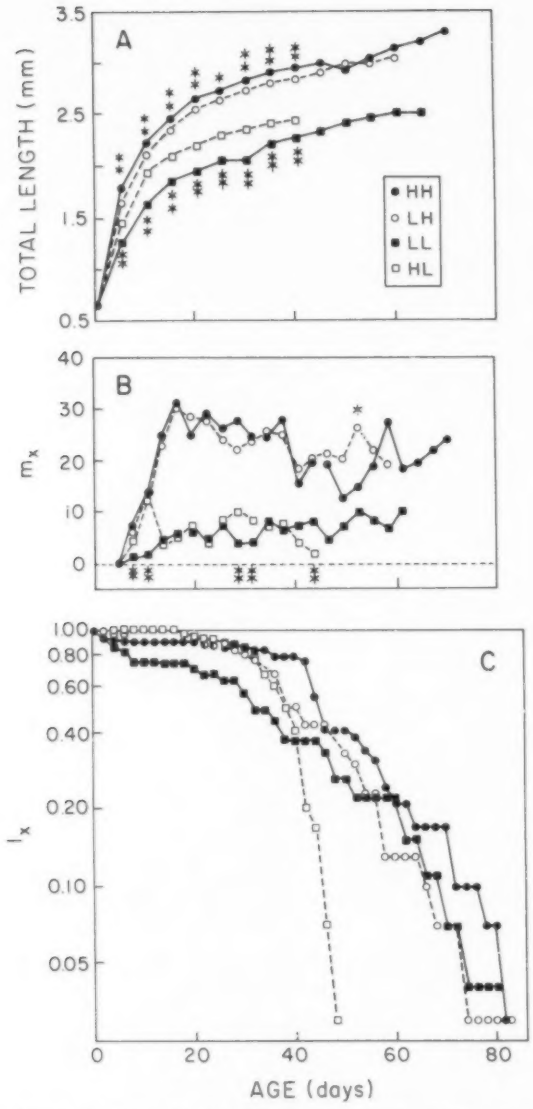

FIG. 2. Growth, reproduction, and survivorship schedules for the four lines. (Data for body size and m_x are only plotted when the sample size ≥ 5.)

A. Significant body size differences between high (HH, LH) and between low (HL, LL) lines are denoted by * ($p < 0.05$) or ** ($p < 0.01$) (t test with unequal sample sizes); all comparisons across high and low food treatments were significant.

B. m_x is the average number of offspring released over a three-day interval; significant differences between low and between high food lines are indicated by * ($p < 0.05$) or ** ($p < 0.01$) (t test with unequal sample sizes). The reproductive output of HL individuals was not significantly different from that of LH individuals at either 7.5 or 10.5 days or from that of HH individuals at 10.5 days, but HH and HL individuals were significantly different ($p < 0.05$) at 7.5 days. All other comparisons between high (HH, LH) and low (HL, LL) lines exhibited significant differences.

C. Comparisons of the survivorship (l_x) schedules are provided in Table 1.

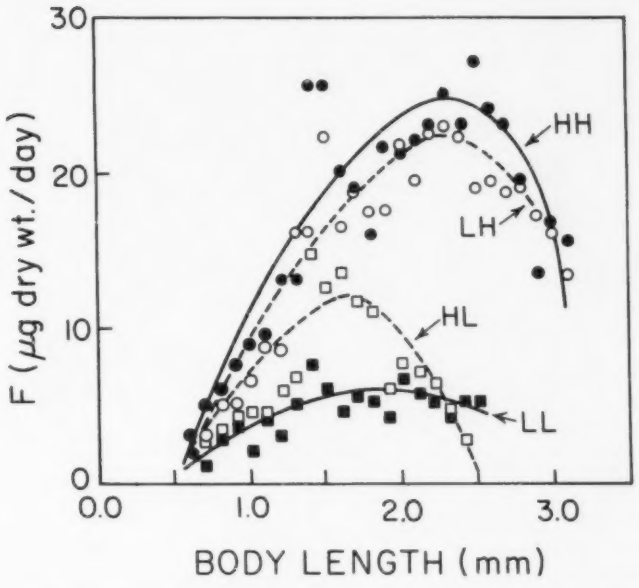

FIG. 3. Mean values of net energy intake, F, for 0.1 mm size classes for the four lines fitted by eye.

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energy intake invested in reproduction by individuals of different sizes. Defining reproductive effort (RE) as the dry weight invested in eggs per instar ÷ the total investment in eggs and growth per instar, the size-specific patterns are virtually identical for all four treatments (Fig. 4). Divergences from the general pattern only appear in those large size classes that occur late in life when survivorship is falling.

These results suggest that size-specific, relative allocation of resources to reproduction is an extremely conservative character for this *Daphnia pulex* clone. However, while reproductive effort has almost always been defined as above or in a very similar manner by ecologists, such a definition does not adequately portray the costs of reproduction for organisms with different energy intakes. A 50% investment in reproduction has much

TABLE 1. CHI-SQUARE COMPARISON OF AGE-SPECIFIC SURVIVAL.

Age (days)	LL vs. HL	HH vs. LH	HL vs. LH	LL vs. HH
10	0.01	NS	NS	0.01
20	0.01	NS	NS	0.01
30	0.01	NS	NS	0.01
40	NS	0.01	NS	0.01
50	0.01	NS	0.01	0.01
60	–	NS	–	NS
70	–	NS	–	NS
80	–	NS	–	NS

Chi-square comparison of l_x between various treatments. NS indicates $p > 0.05$.

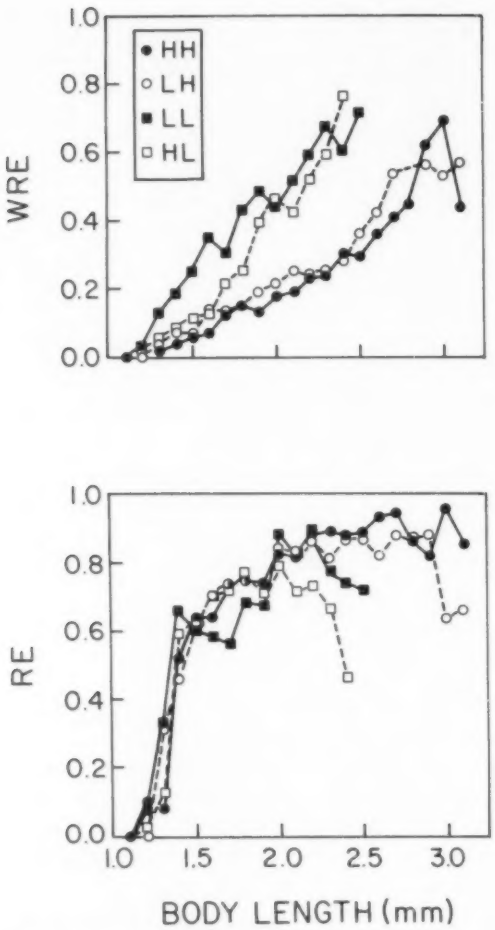

FIG. 4. Mean values of reproductive effort (RE) and weighted reproductive effort (WRE) for 0.1 mm size classes for the four lines.

more significant consequences for the growth and maintenance of a poorly nourished individual than for a well-fed individual (for whom the 50% remaining for growth and maintenance will be much more substantial in absolute terms). This interpretative problem can be rectified by weighting the standard reproductive effort expression (RE) by the absolute investment in growth so that the new measure will give progressively lower values for individuals with equivalent RE but increasing total energy intakes,

$$\mathrm{WRE} = \frac{\mathrm{R}}{(1 + \mathrm{G})(\mathrm{R} + \mathrm{G})} = \frac{1}{1 + \mathrm{G}} \cdot \mathrm{RE}$$

where G and R represent the dry weight investments in growth and reproduction per unit time. Note that WRE = RE only when G = 0.

When viewed in this manner, it becomes apparent that the costs of reproduction are much more severe for the low food lines and become increasingly moreso as the individuals get larger (Fig. 4). Although the weighted reproductive effort function is virtually identical for both high food lines irrespective of maternal environment, there are important differences between the low food lines. While WRE was consistently higher for the LL line than for the high food lines, the HL line started out life with low WRE values similar to the high food lines, and then after the formation of the first two clutches rapidly converged on (but did not surpass) the high WRE values of the LL line. Thus, neither the RE nor WRE sets of data support the hypothesis that the elevated mortality of the HL line is a consequence of an elevated cost of reproduction. Moreover, the general absence of significantly negative correlations between WRE and longevity of individuals within lines (Table 2) indicates that variation in life-span within lines is not a function of variation in reproductive effort.

A closer examination of the total size-specific rates of investment in growth and reproduction suggests an alternative mechanism. F increases and then decreases with size (and age) in all four lines, but the peak comes exceptionally early and the subsequent decline is exceptionally severe in the HL line (Fig. 3). A negative energy budget is indicated for HL individuals larger than 2.5 mm, precisely the size at which the precipitous increase in mortality occurred in this line. This suggests that rather than being a consequence of reproductive effort, the reduced longevity of the HL line resulted from a general breakdown in the physiological capabilities of large individuals. Similar but later and less severe declines in F for the three longer-lived lines suggests that this may be a general mechanism of senescence in this organism.

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Further evidence that the reduced longevity of the HL line is related to its early decline in net energy intake derives from the strong positive phenotypic correlations between $\bar{F}$ in instars 6–10 (~13–24 days) and 11–15 (~25–36 days) and longevity of individuals within this line (Table 2). These correlations were not significant for any of the other lines, but a significant positive correlation did exist between longevity and $\bar{F}$ at a later age (instars 16–20, ~37–48 days) in the HH line. The failure of the LL and LH lines to show similar correlations through the 20th instar does not appear to be an artifact of small sample sizes since these were similar to those for the HH line. However, the decline in F was mildest for these two lines, and it is possible that the correspondence between F and longevity does not arise until later in life when our sample sizes were prohibitively small.

Several differences in phenotypic correlations between the lines indicate that slight differences in parental investment at the egg stage alone can have profound and long-lasting effects on the organization of genome expression in these organisms. For instance, there is a highly significant negative correlation between age at maturity and longevity and positive relation between juvenile growth rate and longevity in the HL line, but no such significant correlations in the other lines (Table 2). Only the LL line exhibits a significant (positive) correlation between progeny production per day and longevity, and only individuals whose mothers were raised on low food exhibit a correlation between size at birth and longevity.

A major question that remains concerns the extent to which the mortality schedules of the different lines are set by events early in post-embryonic development. In particular, are the HL lines committed to an early death by the time juvenile development has been completed because of irreversible physiological and morphological modifications of the phenotype, or can subsequent modification of the environment promote greater longevity?

TABLE 2. CORRELATION WITH LONGEVITY.

Line	Size at Birth	Age at Maturity	Juvenile Growth Rate	Net Energy Intake				Weighted Repro. Effort			
				1	2	3	4	1	2	3	4
HH	0.11	0.17	−0.23	−0.17	0.08	0.28	0.65**	−0.39*	−0.33	−0.22	−0.12
HL	0.26	−0.47**	0.53**	−0.20	0.64**	0.59**	−0.23	−0.29	0.43*	0.05	0.58
LL	0.49**	−0.16	0.17	0.39	0.40	0.30	0.18	0.11	0.23	−0.01	0.37
LH	0.43*	−0.02	0.05	0.13	−0.19	0.07	−0.34	0.10	−0.13	−0.31	−0.30

Correlation coefficients (r) between life span and other life history parameters within the four lines. ** and * indicate significance at the 0.01 and 0.05 levels. Net energy intake and weighted reproductive effort values were squared prior to the regression analysis in order to normalize them.

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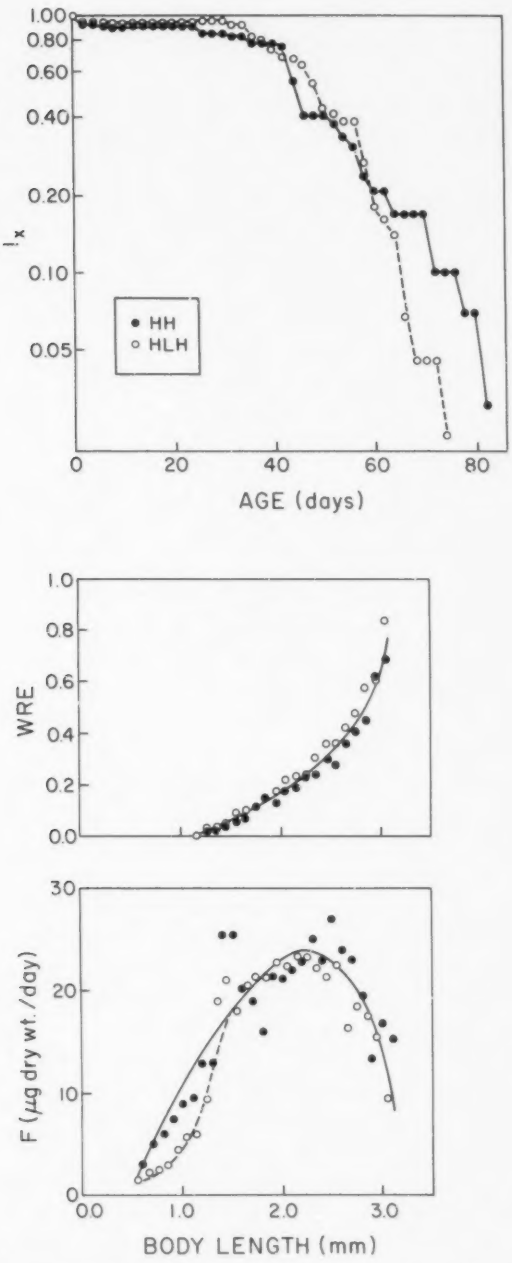

FIG. 5. A comparison of the HLH and HH lines for age-specific survival (l_x) and size-specific weighted reproductive effort (WRE) and net energy intake (F). Chi-square analysis revealed that there were no significant differences in survivorship at the 0.05 level.

We examined this problem by initiating another HL line under identical conditions to the first except for switching all individuals back to high food on their seventh day (approximately their age at maturity).

Figure 5 illustrates that the survivorship schedule of this new line (designated HLH) is statistically indistinguishable from that of the HH line. The size-specific patterns of WRE for the HLH and HH lines are also indistinguishable, and the patterns of net energy intake for the two lines converge following the switch in food regimes for the HLH individuals. Thus, the manifestation of a maternal influence is not simply a function of an individual's setting during its immature phase; it may be highly modified by events occurring after maturation. At the very least then, any prediction of the life-span of an individual requires information on its maternal, pre-reproductive and reproductive environments.

DISCUSSION

We have not established the molecular basis of the maternal effects observed in this study. Goulden and Hornig, 1980 have pointed out the beneficial effects that lipid storage may have on a daphnid's ability to survive during periods of low food availability and have suggested that transfer of lipid stores through eggs may extend the benefit to the progeny. However, our results indicate that maternal stores do much more than simply enhance juvenile survival. Under limiting food conditions, individuals that are endowed with a supplemental store of energy and/or nutrients at the egg stage exhibit an enhanced incorporation of energy into early growth and reproduction far in excess of what can be directly attributed to maternal stores. Moreover, the physiological and/or morphological consequences of maternal investment that increase progeny fitness early in life can be associated with a substantial decrease in survivorship of those same progeny later in life (the HL line). Thus, depending on the environmental setting of the progeny, a small increment in maternal investment at the egg stage alone can result in a radical modification of offspring phenotype.

Since all of the *Daphnia* in this study shared identical genomes, it seems most likely that the observed differences between the four lines are products of the same gene complexes, the outward expressions of which are molded by environmental circumstances. It is possible that the divergence between the HL and LL lines resulted from the expression of different genes whose translation or transcription depended upon their early (embryonic) environmental setting, but the extreme similarity of life histories of the HH and LH lines argues against this. Therefore, our discussion will focus on nongenetic causes of variation in longevity.

Our results are clearly inconsistent with the prominent "cost of reproduction" hypothesis for longevity (Williams, 1966; Calow, 1979). The projected cost of reproduction was exceptionally high for the LL line throughout life, yet its maximal life-span was identical to that of the HH and LH lines. The cost of reproduction for the HL line was relatively low early in life and only approximated that of the LL line later in life, but at the point when all of the HL individuals had died, 25% of the LL individuals were still alive. Finally, of 16 regressions between age-specific WRE and life-span made within lines, only two are significant, one of which is positive (Table 2).

As it is rather loosely defined, the "rate of living" hypothesis for aging does not adequately explain our results either. First suggested by Pearl (1928), the idea that an en-

vironmental reduction of the growth and/or maturation rate will increase longevity is consistent with numerous studies (Ingle, 1933; McCay and Crowell, 1934; McCay *et al.* 1939, Lints and Lints, 1971; Kent, 1981), although cause and effect have not been discriminated in most cases. In our study, however, the relation between juvenile growth rate and lifespan within the lines was insignificant except in the case of line HL for which there was a significantly positive correlation (Table 2). Moreover, when all four lines are compared, a strong positive, rather than negative, correlation between developmental rate and mean longevity is revealed (Fig. 6), a pattern which nonetheless can be completely obviated by modification of the environment following maturation (cf. the HLH line).

Our results are of particular interest when compared to a similarly designed experiment performed by Ingle *et al.*, 1937. They determined life tables for a clone of *Daphnia laevis* on a manure infusion medium (their "well-fed" conditions) and on the same medium diluted 36-fold with pond water (their "starvation" conditions). (Their low-food individuals were actually reproductively active and not really starving to death.) Since both lines were started from "large broods produced by healthy vigorous mothers" (presumably under "well-fed" conditions), they would seem to be analogous to our HL and HH treatments. Yet contrary to our results, Ingle *et al.*'s, 1937 "HL" lines survived longer than their "HH" lines both in terms of mean (38 vs. 28 days) and maximum (51 vs. 46 days) longevity (Fig. 8). A similarly designed experiment later performed with the same clone (Fig. 8) gave comparable results (Dunham, 1938).

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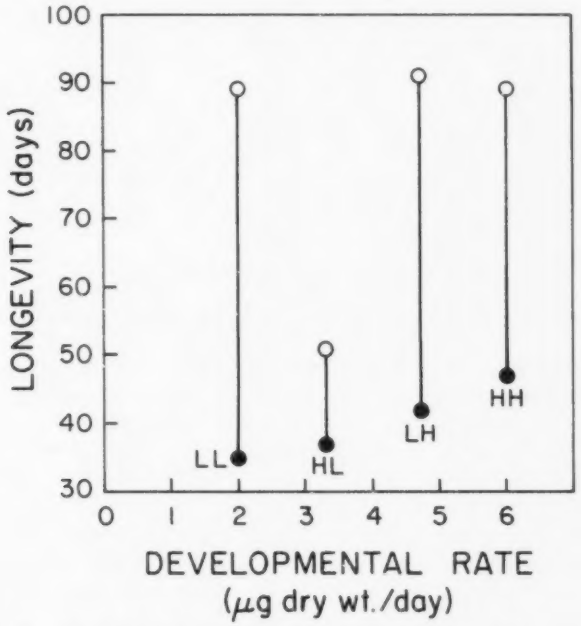

FIG. 6. Mean (solid points) and maximum (open points) life spans and developmental rates for the four lines. Maximum life span was taken to be the age at which $l_x = 0.01$. Developmental rate is equal to the difference in dry wt. between the sizes at maturity and birth divided by the age at maturity.

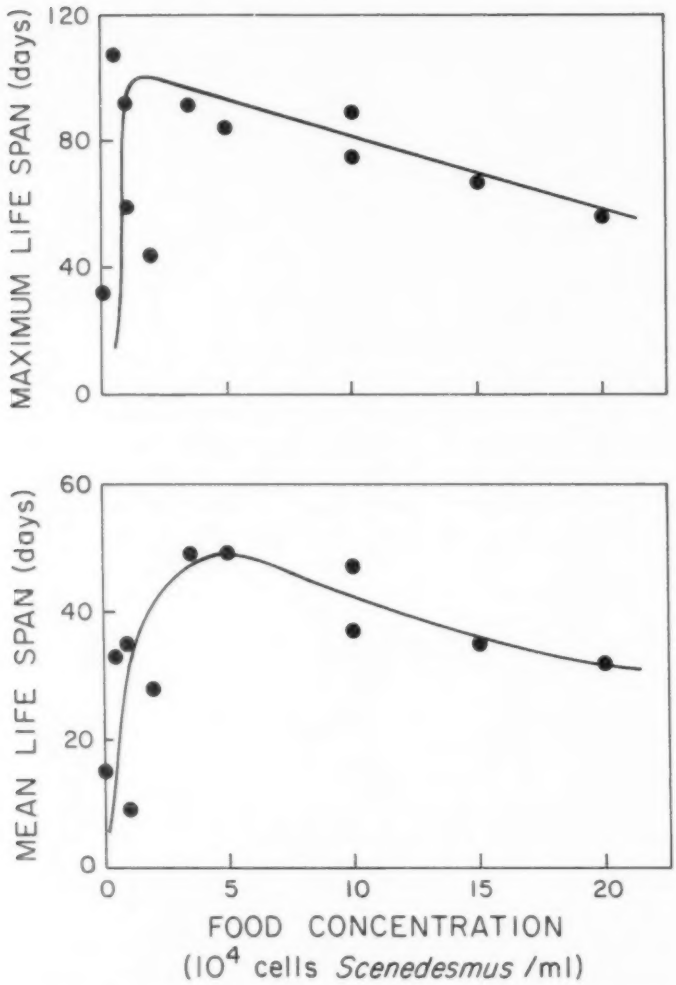

FIG. 7. Mean and maximum longevity as a function of food availability for the *Daphnia pulex* clone used in this study under conditions of identical maternal and progeny environments. Data points include the LL and HH lines of this study as well as nine other lines of 50 individuals each from earlier unpublished work. Maximum life span is the extrapolated age at which $l_x = 0.01$. Food concentration is given as cell density of *Scenedesmus*, but *Chlamydomonas* were also present at one-fifth the *Scenedesmus* level in all cases. Curves fitted by eye.

Rather than being a consequence of a true biological difference between experimental organisms, the discrepancy between our results and those of Ingle *et al.* and Dunham may be an artifact of scale reflecting the non-linear response of longevity to food concentration. In our *Daphnia pulex* clone both mean and maximum life span gradually increase with decreasing food availability until a critical food concentration is reached at which point longevity rapidly declines to zero (Fig. 7); data for other daphnids (Frank *et al.*, 1957; Vijverberg 1976; Porter and Orcutt, 1980) are consistent with this pattern. Thus, the relative life-spans of lines grown on different food concentrations depend on the positions

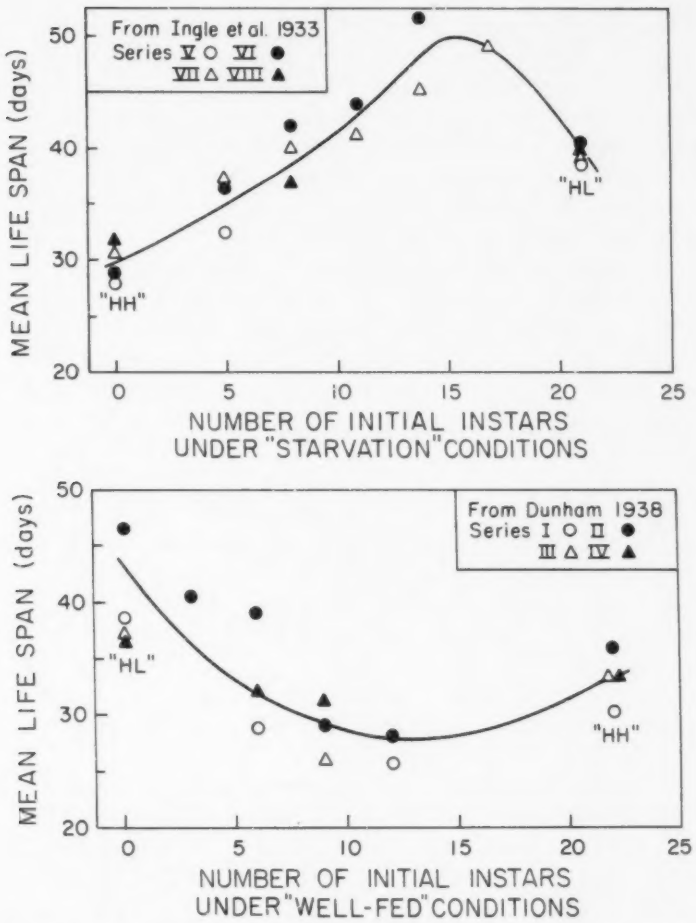

FIG. 8. Mean longevity for lines of a *Daphnia laevis* clone with various schedules of high and low food throughout life. Each point refers to a line of multiple individuals; different series refer to different sets of experiments. Curves fitted by eye.

of those concentrations relative to the optimum for longevity. Our low and high food concentrations straddled the optimum so that the LL line was low on the mean life-span scale relative to the HH line but nearly identical to the HH line on the maximum life-span scale. Although there is no way of translating the food of Ingle *et al.*, 1937 and Dunham, 1938 onto our scale, it is clear that if both their "well-fed" and "starvation" food conditions were in excess of the optimum, a comparison of "HH" and "LL" lines would have shown the latter to have greater longevity. They of course followed an "HL" and not an "LL" line, but the different responses to HL treatment that arose in our studies may be a consequence of the relative positions of H and L foods as well.

One point on which we are in agreement with Ingle *et al.*, 1937 and Dunham, 1938 as well as with other investigators (McCay 1952; Ross *et al.*, 1976; Driver and Cosopodiotis, 1979; Weindruch and Walford, 1982) is that the age of onset of senescence is highly sen-

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sitive to modification by temporal patterns of nutritional conditions (not including starvation) within an individual's life. However, our finding that the longevity of an HL line can be elevated by returning the line to high food at an early age is not entirely consistent with Ingle *et al.*'s (1937) results from raising numerous "HLH" lines with the initial L phase of varying length. Their data show that any initial period on low food elevates longevity with respect to pure "HH" lines, but that only relatively late switches result in an enhancement over "HL" lines (Fig. 8). Again this discrepancy may be a consequence of the different relative food concentrations in our experiments.

Of additional interest is Dunham's, 1938 experimentation with "HHL" lines (Fig. 8). His results indicate that any early period of life spent on high food subtracts from longevity compared to an "HL" line, and suggest that the reduction is greatest at some intermediate number of initial instars spent on high food.

Similar complexities revealed by experimental manipulation of temperature schedules in *Drosophila subobscura* (Clarke and Maynard Smith, 1961) led Maynard Smith (1963) to modify the "rate of living" model to a threshold model. He suggested that the rate of aging (the decline in "vitality") is approximately independent of temperature and that variation in life-span is largely a consequence of variation in the environmentally-determined "threshold vitality" necessary for self-maintenance. Subsequent experimentation with *Drosophila* has yielded data that are not entirely consistent with the threshold model (Hollingsworth, 1969; Lamb, 1968). Moreover, a simple threshold model cannot explain why HLH lines can survive longer than either HL or HH lines or why HHL lines do not survive as long as HH or HL lines. Nor can it explain why longevity increases and then subsequently declines with increasing food availability.

Although neither the "rate of aging" nor "threshold" models are independently capable of explaining longevity variation, and although both concepts must be loosely defined in an absence of a detailed understanding of the mechanisms responsible for senescence at the organismal level, a joint consideration of the two ideas is conceptually useful enough to yield predictive models for life span (Lamb, 1977). In the following analyses, we consider the age at death of an individual to be a function of the level of vitality at birth (V_o), the rate of decline in vitality (the rate of aging, m), and the threshold vitality (V_T). For a constant environment the aging function is defined as

$$v(t) = V_o - mt$$

where v(t) is the vitality at time t. The predicted life span (T) is the age at which v(t) declines below V_T,

$$T = \frac{V_o - V_T}{m} \qquad (1).$$

All three terms on the right can be considered to be functions of maternal (f_m) and/or offspring (f) food supplies.

This simple model immediately helps clarify the conditions necessary to generate a convex relation between life-span and food availability (as in Fig. 7). In a constant environment, mothers and daughters are exposed to identical food concentrations, i.e., $f = f_m = \hat{f}$,

and the behavior of $T(\hat{f})$ can be examined by taking the first derivative of (1),

$$\frac{dT}{d\hat{f}} = \frac{m \cdot \frac{dV_o}{d\hat{f}} + \left\{ - m \cdot \frac{dV_T}{d\hat{f}} - (V_o - V_T) \cdot \frac{dm}{d\hat{f}} \right\}}{m^2}$$

It seems reasonable to assume that vitality at birth is an increasing function of food availability, i.e., $dV_o/d\hat{f} > 0$, particularly since egg volume increases with food availability. Thus, since both $-m$ and $-(V_o - V_T)$ must be negative, the only way for T to first increase and then decrease with $\hat{f}$ (i.e., for $dT/d\hat{f}$ to change sign) is for $dV_T/d\hat{f}$ and $dm/d\hat{f}$ to be of opposite sign. For $T(\hat{f})$ to be convex, the rate of aging must increase and the threshold vitality decrease with increasing food level, or vice versa. We can eliminate those mechanisms that simultaneously increase or decrease m and V_T as primary determinants of $T(\hat{f})$ in environments of constant food availability.

The question then remains as to whether it is the rate of aging or the threshold that increases with food level. Some insight into this problem can be gained from a comparison of the model's predictions with the results of Ingle *et al.* (1937) and Dunham (1938). Since all of their experimental lines were initiated with mothers grown under identical conditions, V_o is a constant, and the differences between lines are entirely functions of the progeny's food schedule, i.e., we can exclude maternal effects as a source of between-lines variance. Fig. 9 graphically portrays the expected patterns of longevity variation in "HLH" and "HHL" lines for the cases in which $dm/df > 0$ and $dV_T/df < 0$ (Model I) and $dm/df < 0$ and $dV_T/df > 0$ (Model II). We see that Model II predicts a decline and subsequent increase in mean longevity as the initial L phase is extended in HLH lines and an increase and subsequent crash in mean longevity as the initial H phase is extended in HHL lines; both predicted patterns are inconsistent with the data of Ingle *et al.* (1937) and Dunham (1938). Model I, on the other hand, generates expected patterns that are qualitatively similar to both sets of data in Fig. 8. This suggests that the rate of aging increases while the threshold vitality declines with increasing availability of food to individuals (f). One possibility is that somatic mutations, metabolic oxidants, autoimmunity and/or some other precursor to cellular damage increases with increasing food availability and the resultant increase in cellular metabolism and mitotic activity, while the tolerance of the organism to specific levels of such damage (i.e., reductions in "vitality") also increases.

This analysis tells us nothing about the mechanistic relation between maternal food availability and an individual's longevity; we cannot simply assume the qualitative effects of f_m and f to be the same. Even our own data which clearly indicate that the maternal environment plays an important role in determining an individual's life-span are insufficient to verify the underlying mechanism. In fact, the patterns observed in Fig. 7 could actually be a consequence of $dm/d\hat{f} < 0$ and $dV_T/d\hat{f} > 0$ if the underlying effects of maternal investment were opposite to and of greater significance than those of the progeny environment. An examination of (1) demonstrates that $\partial V_o/\partial f_m > 0$, $\partial V_T/\partial f_m < 0$ and $\partial m/\partial f_m < 0$ could all be involved in the enhancement of mean longevity that we found with increased maternal investment (Fig. 6). Indeed, one or two of these effects could actually be of opposite sign, and the pattern in Fig. 6 still emerge, if the remaining effects were of substantial magnitude and in the appropriate direction.

Some insight into this matter may be gained from considering the mean and maximum

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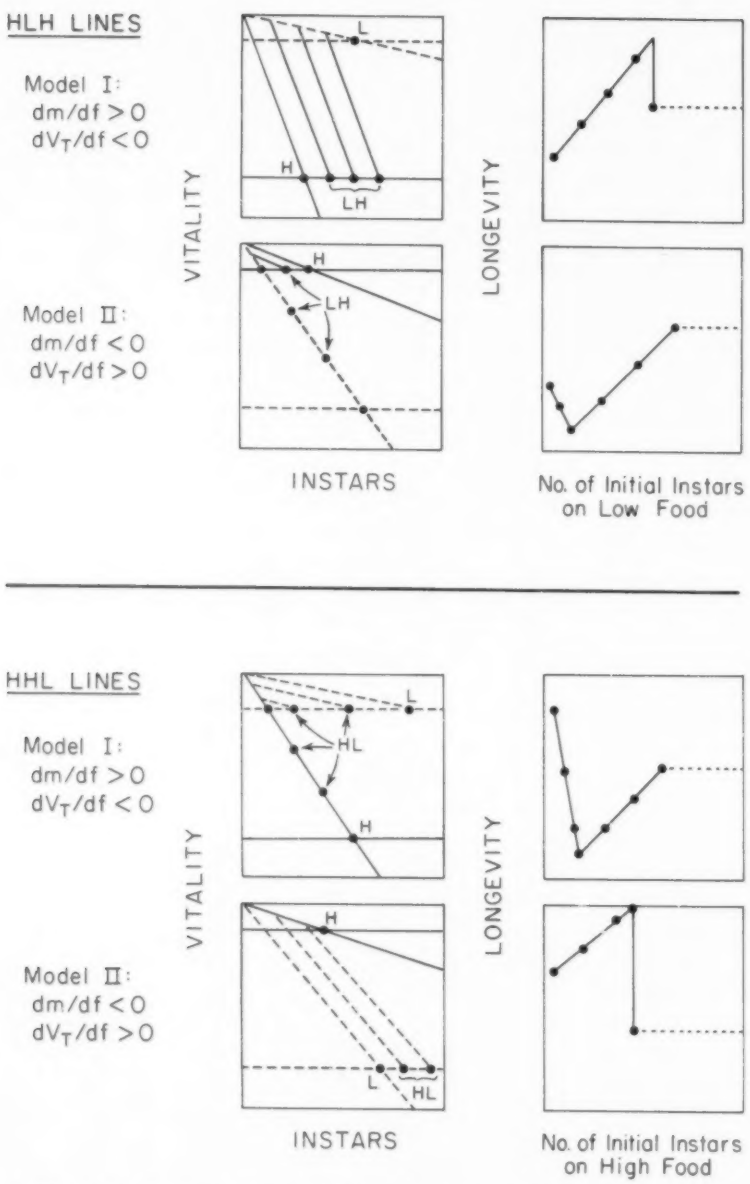

FIG. 9. Expected longevities for lines with various schedules of low and high foods during their lives graphically analyzed with a "rate of aging-threshold vitality" model. Two models are given as explained in the text, and the analysis is applied to lines that are grown on low food early in life and then switched to high food (HLH) and vice versa (HHL). Graphs on the left illustrate the analysis: diagonal lines represent the decline in vitality with age, horizontal lines represent the threshold vitality below which death occurs; dashed lines refer to low food conditions, solid lines to high, and solid points to age at death; L and H lines spend their entire lives on low and high foods respectively, LH and HL have food regimes altered at some point during their lives. Mortality occurs when vitality falls below the threshold for the environment in which the individual is currently living.

longevities of the four lines in our experiment (Fig. 6). For both the low and high lines, increased maternal investment increased the mean but decreased the maximum progeny longevity as a consequence of a decrease in the variance of longevity. An increase in m should tend to synchronize the age at death for a cohort of individuals as they will all cross the vitality threshold in a narrow range of time, and a simultaneous increase in the level of vitality at birth could increase the mean while decreasing the maximum longevity (Fig. 10). Although we are not yet able to determine whether the threshold vitality is also a function of f_m, we tentatively conclude that an increase in maternal investment enhances the quality of offspring at birth (V_o) while simultaneously altering the rate of aging perhaps in a fundamentally similar manner as f.

It is important to keep in mind that the model presented above is intended to explain variation in physiologically determined longevity. It is not meant to explain the life-span of individuals in many natural populations that are exposed to selective agents (such as predators) that can impose mortality prior to the depression of "vitality" below the physiological threshold. Moreover, it is perhaps premature to generalize our experimental findings to other organisms. For instance, while the results of Robertson and Salt (1981) using the rotifer *Asplanchna girodi* are qualitatively similar to the results of Ingle *et al.* (1937) and Dunham (1938) for mid-life shifts in food availability, the results of their maternal effects experiment are fundamentally different from our own—an enriched maternal environment either had no effect or a deleterious effect on mean life-span. While ex-

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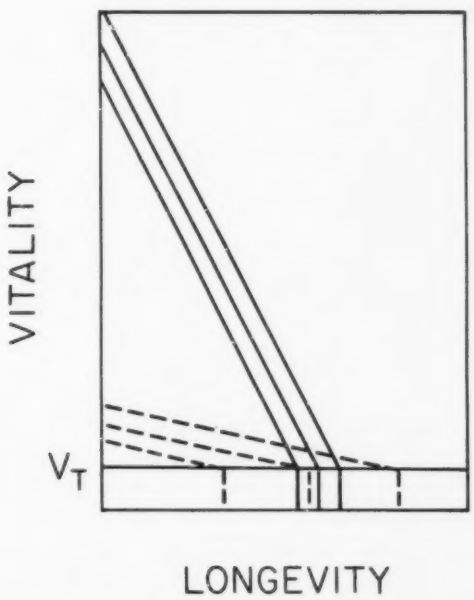

FIG. 10. Mean and range of expected life spans for two lines grown on identical food supplies but with different maternal effects. Dashed lines represent individuals whose maternal environment was low food, solid lines represent high food maternal environments. It is assumed that an increase in the maternal food supply improves the vitality at birth but increases the rate of ageing. An individual's life is terminated when its vitality declines below the threshold, V_T.

perimentation with rats, mice and hamsters (McCay 1952; Stuchlíková *et al.*, 1975) is in complete agreement with the *Daphnia* data that indicate that dietary restriction early in life followed by complete diet is especially conducive to long life-span, results with rodents also indicate that the initiation of dietary restriction late in life enhances longevity (Stuchlíková *et al.*, 1975, Weindruch and Walford, 1982) contrary to the results of Dunham, 1938. It remains to be seen whether these discrepancies are a consequence of variation in the mechanisms of aging and/or evolutionary strategies of phylogenetically diverse organisms or simply artifacts of employing food concentrations with fundamentally different locations with respect to the longevity-food availability relation. For instance, both of the food concentrations used by Robertson and Salt (1981) were to the right of the peak of $T(\hat{f})$ while our two concentrations straddled the peak. Future work involving several food concentrations will help resolve this problem.

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ROLE OF METABOLIC RATE AND DNA-REPAIR IN *DROSOPHILA* AGING:

Implications for the Mitochondrial Mutation Theory of Aging

JAIME MIQUEL,* ROSEMARIE BINNARD,* and JAMES E. FLEMING†

*NASA Ames Research Center, Biomedical Research Division, Moffett Field, CA 94035
†Linus Pauling Institute of Science and Medicine, Palo Alto, CA 94306

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Abstract—The respective roles of respiration rate and DNA repair in determining the life-span of *Drosophila melanogaster* have been investigated in three wild strains (Oregon R, Domodedov 32, & Swedish C) and two mutants (w/w & w/mei-41 D5). In agreement with the rate of living theory, there was an inverse relation between oxygen consumption and median life-span in flies having normal DNA repair. In contrast, w/mei-41 D5, showed an abnormally low life-span as compared to the controls. The median life-spans for these flies were: Oregon R, 49; Domodedov 32, 46; Swedish C, 35; w/w, 33; and w/mei-41 D5, 22. Furthermore, this mutant also showed significant deficiency in mating fitness and a depressed metabolic rate. These data suggest that the short life-span of the w/mei-41 D5 is a consequence of a specific syndrome unrelated to normal aging and is not the expression of accelerated senescence. It is proposed that DNA repair mechanisms influence adult life-span by their effect on actively dividing progenitor cells during development rather than by modulating sensescence of the resulting differentiated cells of the imago during adulthood.

INTRODUCTION

WE HAVE PROPOSED previously that the mitochondria of differentiated cells may be the site of intrinsic aging because of attack by free radicals and lipid peroxides originating in the organelles as a by-product of the reduction of oxygen during respiration (Economos *et al.*, 1980; Miquel *et al.*, 1980, Fleming *et al.*, 1982). In our concept the site of senescent injury is the mitochondrial DNA (mtDNA) rather than the biomembranes, which in the views of Harman (1956, 1962, 1972) and Tappel (1965) play the main role as the targets of uncontrolled lipid peroxidation leading to cell aging and death. Specifically, we assume that during maturation of the organism an increasing number of mitochondria lose the ability to divide as a consequence of mtDNA injury and thus become vulnerable to progressive membrane damage because of lipid peroxidation and cross-linking. Our mitochondrial DNA mutation (or inactivation) theory of cell aging is in agreement with the following experimental data: 1) normal senescence is accompanied by mitochondrial alteration and loss and by lipid peroxidation of mitochondrial membranes resulting in the genesis of

Correspondence should be addressed to J.E. Fleming.

fluorescent lipopigments (Miquel *et al.*, 1977; Miquel *et al.*, 1980); 2) increased mitochondrial respiration in *Drosophila* exposed to moderately high temperatures results in both higher rates of lipopigment accumulation and life shortening (Miquel *et al.*, 1976); 3) adult *Drosophila* showing lower respiration rates because of exposure to the mtDNA-intercalating dye ethidium bromide during development live longer than controls raised on normal medium (Fleming *et al.*, 1981); 4) aging results in a striking loss of mitochondrial DNA in *Drosophila melanogaster* while the quantity of nuclear DNA remains constant (Massie *et al.*, 1975, 1981); 5) there is an inverse correlation between oxygen consumption and life-span in *D. melanogaster* treated with high levels of dietary antioxidants (Miquel *et al.*, 1982).

In view of the above, we predict that the different respiration rates shown by several wild strains of *Drosophila* maintained in our laboratory would be accompanied by inversely proportional life-spans. This concept was tested in wild flies of the Oregon R, Swedish C, white eyed (w/w) and Domodedov-32 strains. In addition, since nuclear DNA-repair is positively correlated with life-span in mammalian species (Hart and Setlow, 1974; Hart *et al.*, 1979), we thought it of interest to investigate respiration rate and aging in a mutant of *D. melanogaster*, namely the *w mei*-41^{D5}, which is deficient in postreplication repair as shown by its reduced capacity to synthesize DNA following UV irradiation of primary tissue cultures derived from embryonic cells (Baker and Carpenter, 1972; Baker *et al.*, 1972, 1978; Boyd and Setlow, 1976; Boyd *et al.*, 1980). The specific reason for the use of this mutant was to test if its repair deficiency resulted in a life-span that was shorter than that predicted on the basis of its O_2 consumption.

MATERIAL AND METHODS

Oregon R wild type *Drosophila melanogaster* were from a stock that has been maintained in our laboratory for over 20 years. Swedish C fruit flies were obtained from the *Drosophila* Stock Center, California Institute of Technology. The Domodedov-32 strain was obtained from Dr. G.E. Parfenov (U.S.S.R. Institute of Biomedical Problems, Moscow). The DNA-repair deficient *w mei*-41^{D5} flies and their parental white-eyed strain were obtained from the Department of Genetics at the University of California, Davis. All flies were reared and maintained at 25°C on instant medium as previously described by Fleming *et al.* (1981). In addition to mortality kinetics, fly respiration *in vivo* was determined in a Gilson respirometer, as described elsewhere (Fleming *et al.*, 1981). Mating ability was measured by a modification of the method of Economos *et al.*, 1979. Instead of using three virgin females per male in each vial, 15 females and 10 males were housed in each vial and the number of accomplished matings was recorded at 3, 6, 9 and 12 minutes after housing males and females together. The rationale of the selection of median life-span as the most accurate parameter in longevity studies can be found in J.M. Ordy *et al.* (1971).

RESULTS AND DISCUSSION

As shown in Table 1 and Fig. 1, the mean, median and maximum life-spans of the repair deficient strain were considerably shorter than those of the parental strain, the white-eyed (w/w) strain endowed with normal DNA-repair mechanisms. Moreover, while there was an approximately inverse correlation between life-span and O_2 consumption for the three wild strains (Fig. 2), this was not the case for the w mei-41^{D5} strain, which showed abnormally low values for its mean, median and maximum life-span.

The results on the wild strain are in agreement with Pearl's (1928) rate of living theory and with our recent concepts on the key role of respiration-dependent mitochondrial loss in aging (Economos *et al.*, 1980; Miquel *et al.*, 1980; Fleming *et al.*, 1981). The contrasting results obtained with the *w mei*-41^{D5} strain are consistent with the hypothesis that DNA-repair plays an important role in determining the life-span of this *Drosophila* mu-

TABLE 1. LIFE-SPAN, O_2 CONSUMPTION AND MATING ABILITY OF NORMAL WILD STRAINS AND OF A DNA REPAIR-DEFICIENT MUTANT OF *D. MELANOGASTER*.

Strain	No. of flies	Mean life-span, days	S.D.	Median life-span, days	ul O_2 consumption/ mg fly/24hrs.	S.D.	Percent mating*	S.D.
Oregon R	345	48.5	6.7	49	72.2	15.3	80.4	1.5
Domodedov	201	45.8	13.1	46	88.5	21.1	90.9	0.9
Swedish-C	186	35.2	8.9	35	98.9	8.3	41.6	2.4
White-eyed, (w/w)	353	32.0	9.1	33	94.4**	13.7	64.3**	2.2
White-eyed w mei-41^{D5}	369	24.0	6.6	22	86.3**	14.2	20.0**	1.9

*Calculated from the number of males which had accomplished mating at 12 minutes.

**The difference between the (w/w) and the *w mei*-41^{D5} (DNA-repair deficient) males was significant ($p < 0.05$, Students t tests).

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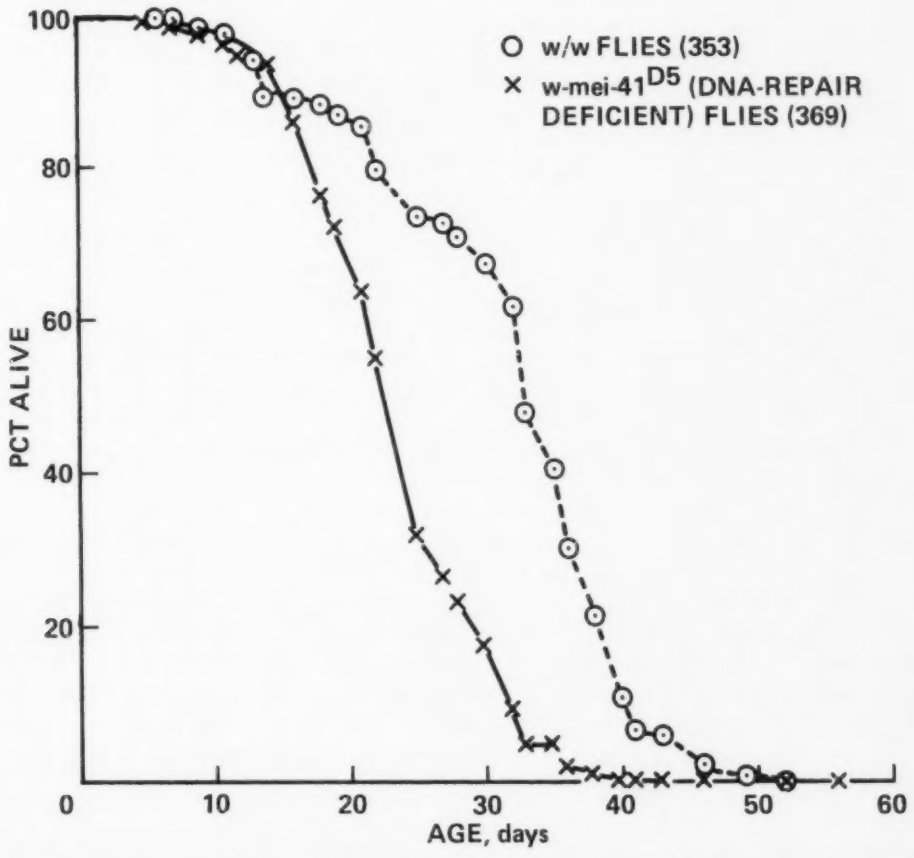

FIG. 1. Survivorship curves of normal and DNA-repair deficient male *Drosophila melanogaster*. Initial number of flies in parentheses.

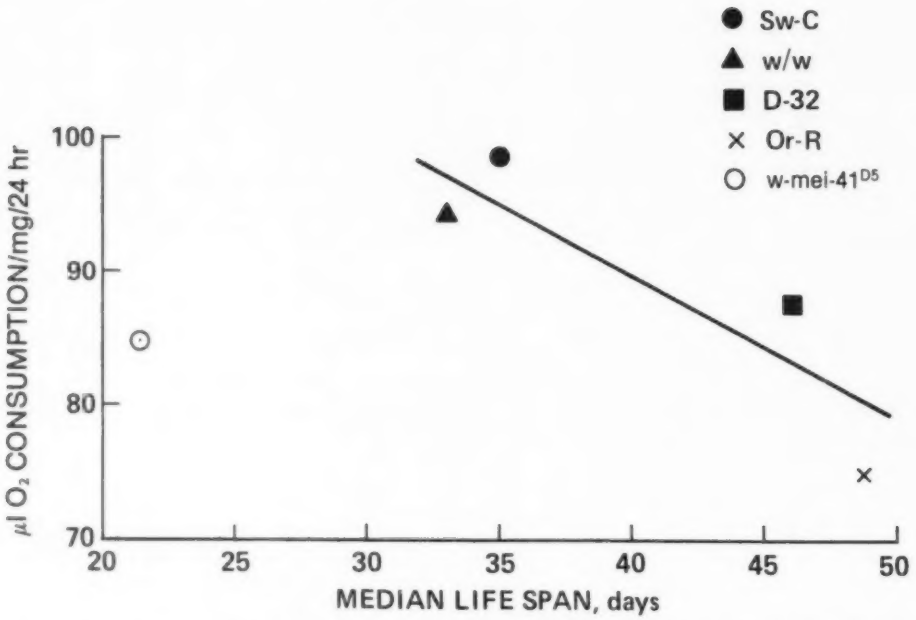

FIG. 2. Oxygen consumption and median life-span of normal and DNA-repair deficient male flies. The DNA-repair deficient (*w mei*-41^{D5}) flies have a shorter life-span than the wild strains, including their parental strain w/w.

tant. A complete interpretation of the present data would require the additional assumption that in the three wild strains DNA-repair (and other longevity-promoting mechanisms) are equally effective and therefore life-span is a simple function of respiration rate. This interpretation of our findings is in agreement with Pearl's concept that the duration of life is determined by two factors: 1) the *inherent vitality* or metabolic potential, which is the result of genetic factors; and 2) the actual metabolic rate of the aging organism. The DNA-repair mechanisms would be one of the constituents of the *inherent vitality*, which counteract, though not with an effectiveness of 100%, the senescence-causing side effects of mitochondrial respiration.

The poor mating performance of the *w mei*-41^{D5} male flies in comparison with all other strains (Table 1) suggests that the mutants are deficient in *inherent vitality* even during the first days of adult life, which are usually associated with maximum vigor. Therefore, the short life-span of the *w mei*-41^{D5} flies may be the result of a specific debilitating "congenital disease" rather than the expression of accelerated aging. In agreement with this interpretation of the experimental data, DNA-repair deficiency in *Drosophila* influences life-span by setting the stage (during embryonic development) for premature failure of the imaginal organs rather than by modulating cell aging during adulthood. On the other hand, the present data provide additional evidence in favor of the pace-setting role of respiration (and concomitant mitochondrial injury) in *Drosophila* aging.

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AGE-DEPENDENT CHANGES OF HORMONE-STIMULATED GLUCONEOGENESIS IN ISOLATED RAT HEPATOCYTES

ZBIGNIEW KMIEĆ* and ANDRZEJ MYŚLIWSKI

Department of Histology, Medical School, 80-210 Gdańsk, Poland

(*Received* 21 *July* 1982)

Abstract—Effects of glucagon or catecholamine stimulation on glucose synthesis from lactate/pyruvate, alanine, fructose or glycerol in isolated hepatocytes from young and old rats were compared. It was found that production of glucose by non-stimulated hepatocytes was almost the same in hepatocytes both from young and old rats. The concentrations of hormones which caused the maximal effect were the same for both compared groups of rats. However, the effect of stimulation by glucagon was significantly greater in hepatocytes of young rats than of old ones, when lactate/pyruvate or alanine were used as substrates, while no such difference was observed when fructose or glycerol were used. Epinephrine and norepinephrine stimulated glucose synthesis from all substrates investigated to the same extent in hepatocytes isolated from both compared groups of rats. The biochemical and physiological aspects of these findings are discussed.

INTRODUCTION

THE MOST SIGNIFICANT feature of aging is reduced ability of the organism to adapt to changing conditions of the environment (Comfort, 1964). This results from a decline in the function of various organs. The impairment of their function may be caused either by changes in cell function itself or the changes in neurohormonal regulation (Frolkis *et al.*, 1970; Andres *et al.*, 1977; Finch, 1977; Everitt, 1980). Recent developments in the methods of cell isolation made it possible to determine which of the above mentioned processes is responsible for the decline of function of a given organ during aging.

Homeostasis of plasma glucose in mammals is most important for the maintenance of brain, peripheral nerve tissue, blood cells and kidney medulla as these tissues rely entirely upon glucose as a source of energy. In normal conditions, the carbohydrate supply in the diet of omnivorous mammals seems sufficient to maintain plasma glucose. However, during starvation and/or other hypoglycemic states, liver gluconeogenesis becomes essential in glucose production after the total exhaustion of glycogen stores. The main glucogenic substrates are lactate, alanine and glycerol (Exton, 1972). Gluconeogenesis is under the precise control of several hormones, among which glucagon, catecholamines, insulin and glucocorticoids play the main role (Scrutton *et al.*, 1968; Exton, 1972).

*To whom all correspondence should be addressed.

Abbreviations—db cAMP dibutyryl cyclic adenosine 3′,5′-monophosphate, cAMP cyclic adenosine 3′,5′-monophosphate.

Although during aging some changes in glucose homeostasis have been observed (Davidson, 1979), there is no data indicating that quantitative response of the hepatocytes to stimulation by a particular hormone is altered. The aim of the investigations reported in this paper was to compare the ability of glucose production from various substrates by hepatocytes isolated from young and old rats upon stimulation by glucagon, norepinephrine and epinephrine.

Two groups of substrates were investigated – pyruvate, lactate and alanine which enter gluconeogenesis in mitochondria, the last two being converted to pyruvate in the cytoplasm, and glycerol and fructose, substrates converted to glucose outside mitochondria through a sequence of enzymatic steps located exclusively in the cytoplasm with a possible involvement of endoplasmic reticulum (Krebs *et al.*, 1976; Veneziale, 1976).

MATERIAL AND METHODS

Animals

Male Wistar rats 6–8 and 20–24 months old ("young" and "old," respectively) were used. They were maintained on commercial stock diet throughout the life span. The animals were fasted for 24 hours before the beginning of hepatocytes isolation. The procedure of hepatocytes isolation always took place between 9.00 and 11.00 a.m.

Isolation of hepatocytes

Hepatocytes were isolated according to own modification (Kmieć *et al.*, 1979) of the method described by Seglen 1975. The animals were anesthetized by an interperitoneal injection of thiobutabarbital natrium (Germed) 180 mg/kg body weight. The first perfusion step with Ca^{++} – free modified Hanks' solution (solution 1, Table 1) was carried out for 2–4 min (temp. 37°C) at a flow rate of 28–30 ml/min and was followed by recirculation step of the Ca^{++} – containing collagenase solution (solution 2, Table 1) at the same rate for 15–25 min (temp. 37°C). The pH value of the enzymatic solution remained between 7.45 and 7.60 and therefore no adjustments were necessary. The washing and purification step of the isolation procedure was performed according to Seglen (1975), at temp. 4°C. Finally 2–4 $\times 10^8$ cells were suspended in 4–6 ml of solution 3 (Table 1) and immediately

TABLE 1. COMPOSITION OF SOLUTIONS USED IN THE ISOLATION AND INCUBATION PROCEDURES.

	mmoles/litre			
Composition	Solution 1 (for pre-perfusion)	Solution 2 (for enzymatic perfusion)	Solution 3 (for filtration and washing)	Solution 4 (Krebs Ringer buffer)
Na Cl	137.0	137.0	137.0	118.0
KCl	5.36	5.36	5.36	4.7
$CaCl_2$	–	5.0	1.27	1.3
KH_2PO_4	0.44	0.44	0.44	1.2
$Na_2HPO_4 x\ 2H_2O$	0.38	0.38	0.38	–
$NaHCO_3$	4.17	4.17	4.17	25.0
Glucose	10.0	10.0	–	–
HEPES	–	40.0	–	–
Collagenase	–	0.05% (w/v)	–	–
Gaseous phase	air	air	air	O_2 + CO_2 /95:5/
pH	7.6	7.6	7.6	7.45[a]

[a]pH was adjusted with 1 M solution of KH_2PO_4 after the solution was gassed with O_2 mixed with CO_2 (95:5) for 20 min.

0.1 ml of that suspension was taken and mixed with 0.9 ml of Krebs-Ringer buffer (solution 4, Table 1) for incubation.

Incubation procedure and analytical methods

Incubation was performed in siliconized 18 cm^3 glass vessels. Incubation mixture contained: 2–4 × 10^6 of cells suspended in 1 ml of Krebs-Ringer buffer (solution 4, Table 1); substrates: lactate, pyruvate, glycerol alanine or fructose and hormones: glucagon, epinephrine or norepinephrine at concentrations indicated. Substrates and hormones were dissolved in solution 3 (Table 1) immediately before use and were added to the incubation vessel as concentrated solution in a volume of 25 µl. After gassing for 3 min with 95% O_2 and CO_2, the vessels were tightly stopped with rubber stoppers and put in metabolic water bath set at 37°C with shaking (90 oscillations in min) – this moment is defined as the beginning. At time intervals indicated, 0.25 ml samples of cell suspensions were taken from each vessel and deproteinized by mixing with 0.05 ml of 20% (v/v) perchloric acid. Precipitated protein was removed by centrifugation for 10 min at 1500g and supernatant was kept for 2 hours at 4°C before glucose determination was performed.

Glucose was estimated in duplicate using 50 µl aliquots of acidic supernatant by the glucose oxidase method according to Werner *et al.*, 1970. Cell protein content was determined by a biuret method (Layne, 1957) using bovine albumin fraction V as standard. Glycogen content of isolated hepatocytes was assayed by the method of Walaas and Walaas, 1959. For the hydrolysing glycogen, 0.25 ml of cell suspension was treated with 0.75 ml 30% (w/v) KOH at 100°C for 40 min. Dry mass of cells was determined as described by Berry and Kun, 1972. The number of cells in suspension was counted in Fuchs-Rosenthal hemocytometer using Türk's solution for dilution.

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Viability and morphology of isolated hepatocytes

The viability of isolated liver cells was determined by mixing 0.1 ml of cell suspension with 0.2 ml of 0.4% (w/v) trypan blue solution; the damaged cells did not exclude stain and remained dark. Only the cell preparations with viability 85% or more were used.

For the ultrastructural studies, 0.5 ml of cell suspension was mildly centrifuged (200g for 1 min) and the cell pellet was fixed for 16–18 hours at 2.5% (w/v) glutaraldehyde at 4°C. The cells were then post-fixed for 60 min in 2% osmium tetraoxide and dehydrated with increasing concentrations of ethanol up to 100%, infiltrated with propylene oxide and Epon 812. After polymerization, solid blocks were sectioned (70 nm) with Reichert Om U2 ultramicrotome. Finally, sections were stained with 2% uranylacetate in absolute ethanol and post-stained with lead-citrate.

The internal ultrastructure of isolated hepatocytes was quite normal in liver cells from both compared groups of rats and corresponded to that described by Berry and Friend, 1969. The only difference at ultrastructural level between hepatocytes of young and old rats was a greater number of "myelin bodies" and lipofuscin granules in cells of old rats.

Materials

Phenoxybenzamine was a kind gift of Smith, Kline and French, dihydroergotamine was a kind gift of Serva. The following reagents were purchased: glucagon from Novo; dibutyryl cyclic 3′,5′-AMP epinephrine, HEPES (N-2-hydrooxylethylpiperazine-N′-2 ethanosulfonic acid), isoproterenol, propranolol, Na-pyruvate from Sigma; L-alanine, D-fructose from BDH; glycerol, Na-lactate from Merck; collagenase from Boehringer; D-glucose from Polfa, Epon 812 from Serva. All other chemicals were of reagent grade and were purchased from POCh, Gliwice, Poland.

Statistics

The results of experiments were estimated statistically with aid of linear regression analysis, paired *t*-Student test and *t*-Student test (Gren, 1974).

RESULTS

Protein content and dry mass of isolated hepatocytes

The results presented in Table 2 indicate that isolated hepatocytes of young and old rats contain very similar amounts of proteins and have almost the same dry mass. Therefore, it was possible to relate the results of the measurements of glucose synthesis to the protein content in hepatocytes.

TABLE 2. PROTEIN CONTENT AND DRY MASS OF ISOLATED HEPATOCYTES.

Age of animals /months/	Number of determinations	Protein content in 10^6 cells (mg)	Dry mass of 10^6 cells (mg)
6–8	15	2.45 ± 0.29	2.37 ± 0.23
20–24	17	2.61 ± 0.31	2.29 ± 0.28

The results are mean ± SEM. The differences of values for adult and old rats were not statistically significant: $p > 0.05$.

Glycogen content in isolated hepatocytes

As shown in Table 3 24 hours fasting is sufficient to cause almost complete depletion of glycogen stores in hepatocytes of both young and old rats. Observed big amounts of glycogen in hepatocytes isolated from nonfasting rats indicate that the procedure of cell isolation applied did not interfere with the stores of glycogen in hepatocytes. Therefore one may assume that glucose appearing in the incubation medium was produced by cells from the substrates provided.

Stimulation of glucose synthesis in hepatocytes of young and old rats by glucagon and catecholamines

a) Effect of hormone concentration: To determine the susceptibility of hepatocytes to stimulation by glucagon, epinephrine or norepinephrine, the cells were incubated with the increasing concentrations of these hormones. The results of these experiments are presented in Figs. 1 and 2. In both animal groups investigated the plot of glucose production versus hormone concentration displayed a shape of saturation curve. Both in young and old rats the maximal stimulation of synthesis, with all substrates investigated was obtained at 5×10^{-6} M of glucagon and 5×10^{-6} M of catecholamine. The extent of stimulation produced by glucagon in whole range of concentrations applied was different in both groups compared when lactate/pyruvate or alanine were used as substrates. With glycerol or fructose the stimulatory effect was the same. Stimulation with epinephrine or norepinephrine caused the same effect in both compared groups for all substrate and hormone concentrations employed.

TABLE 3. GLYCOGEN CONTENT OF ISOLATED HEPATOCYTES.

Age of animals (months)	nmoles of glucose equivalent/mg protein	
	Animals fasted for 24 hours	Animals fed ad libitum
6–8	2–23 (5)	600, 820 (2)
20–24	2–30 (5)	582, 933 (2)

Glycogen determined as described in Methods. Number of cell preparations investigated given in parenthesis.

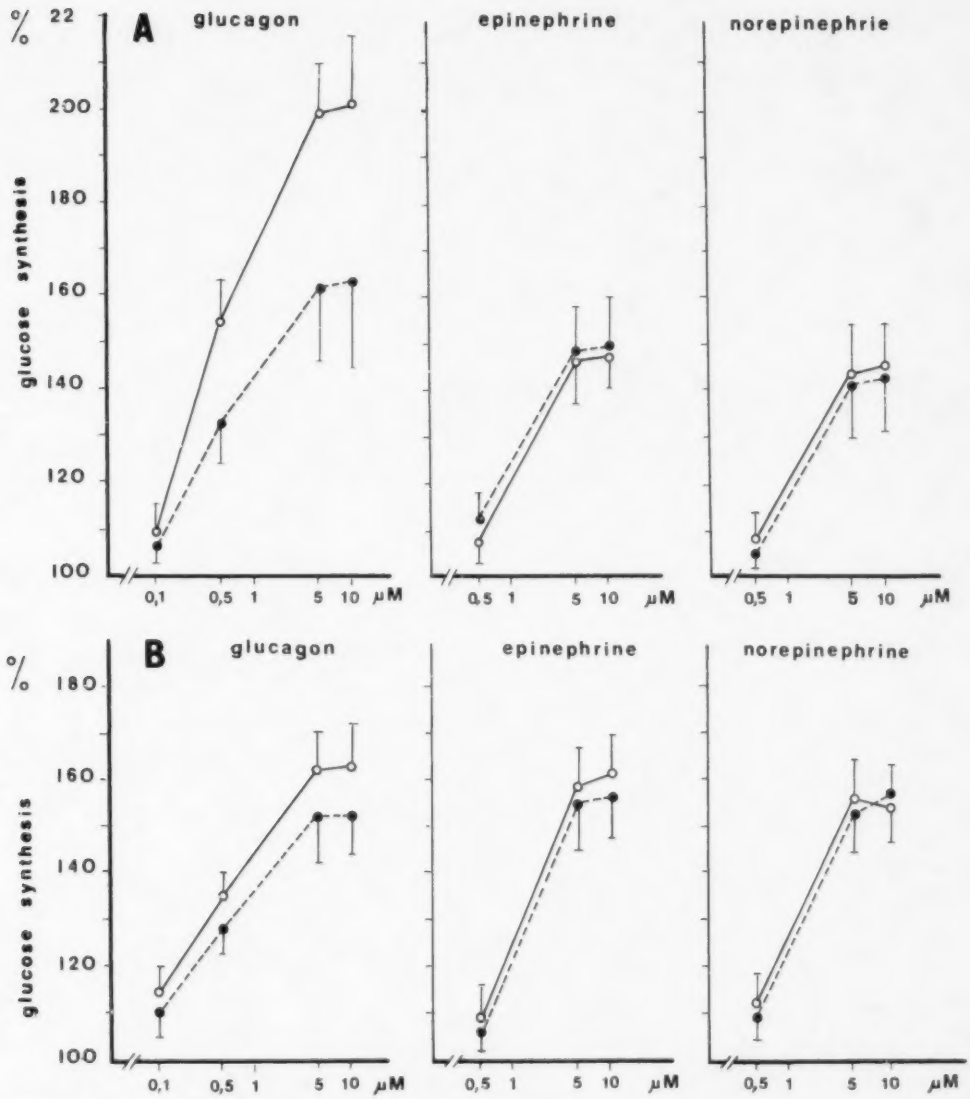

FIG. 1. Effect of hormone concentration on the synthesis of glucose from (A) lactate/pyruvate (18:2 mM) and (B) fructose (5 mM) in isolated hepatocytes of young and old rats. Hepatocytes of young (open circles) and old (closed circles) rats were incubated for 40 min as described in Methods. The results are the means from 5–7 experiments, vertical bars represent SEM. Abscissa—concentration of hormone (in μM). Ordinate—relative glucose synthesis assuming as 100 the glucose production in the absence of hormone added.

b) Glucose synthesis from various substrates in the presence of optimal concentrations of glucagon and epinephrine: The results of experiments with hepatocytes incubated in medium containing glucagon or epinephrine in optimal concentrations are presented in Table 4 and in Figs. 3 and 4. The presented data indicate that the rate of unstimulated glucose synthesis from each substrate investigated was very similar in hepatocytes from both

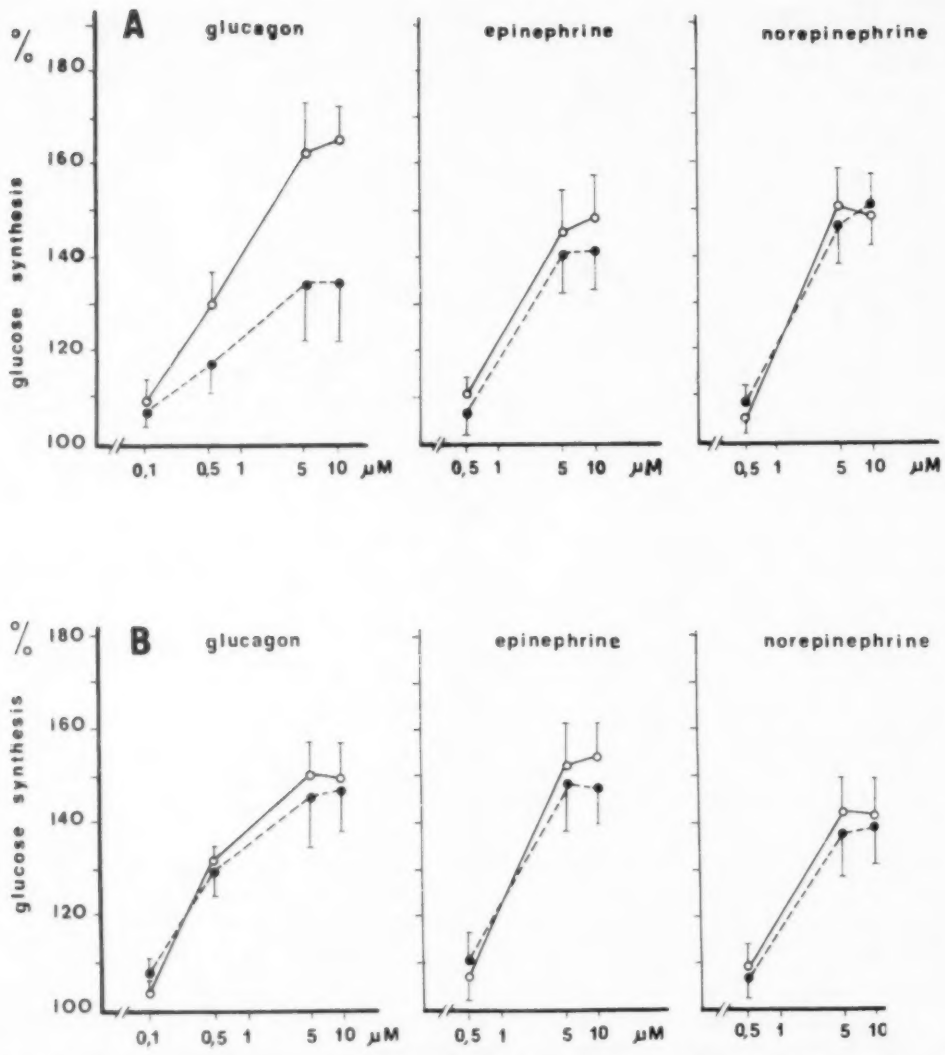

FIG. 2. Effect of hormone concentration on the synthesis of glucose from (A) alanine (10 mM) and (B) glycerol (10 mM) in isolated hepatocytes of young and old rats. Hepatocytes of young (open circles) and old (closed circles) rats were incubated for 40 min as described in Methods. The results are the means from 7–9 experiments, vertical bars represent SEM. Abscissa – concentration of hormone (in μM). Ordinate – relative glucose synthesis assuming as 100 the glucose production in the absence of hormone added.

young and old rats. In all experiments, the observed glucose synthesis was linear during the whole incubation time. In the control experiment performed without exogenous substrate added, only negligible glucose synthesis from endogenous substrates could have been observed.

Glucagon and epinephrine stimulated glucose production from all substrates investi-

TABLE 4. EFFECT OF GLUCAGON AND EPINEPHRINE ON GLUCOSE SYNTHESIS FROM VARIOUS SUBSTRATES IN ISOLATED HEPATOCYTES FROM YOUNG AND OLD RATS.

Substrates	nmoles glucose/min/mg protein		
	No hormone	Glucagon (5 μM)	Epinephrine (5 μM)
Young rats (6–8 months)			
Control—without substrates added	0.20 ± 0.03 (22)	0.23 ± 0.04 (5)	0.21 ± 0.05 (5)
Lactate/pyruvate (18:2 mM)	1.74 ± 0.21 (7)	3.41 ± 0.38 (7)	2.29 ± 0.29 (7)
Alanine (10 mM)	1.26 ± 0.17 (6)	2.07 ± 0.30 (7)	1.73 ± 0.26 (6)
Glycerol (10 mM)	0.91 ± 0.13 (8)	1.41 ± 0.17 (8)	1.48 ± 0.21 (8)
Fructose (5 mM)	4.96 ± 0.96 (6)	7.11 ± 0.97 (6)	6.89 ± 1.06 (6)
Old rats (20–24 months)			
Control—without substrates added	0.23 ± 0.03 (25)	0.24 ± 0.25 (6)	0.22 ± 0.04 (5)
Lactate/pyruvate (18:2 mM)	1.68 ± 0.24 (8)	2.57 ± 0.33[a] (8)	2.35 ± 0.39 (8)
Alanine (10 mM)	1.14 ± 0.18 (7)	1.46 ± 0.18[a] (7)	1.63 ± 0.21 (7)
Glycerol (10 mM)	0.80 ± 0.15 (7)	1.28 ± 0.18 (8)	1.34 ± 0.19 (7)
Fructose (5 mM)	4.67 ± 0.72 (8)	6.53 ± 0.87 (8)	6.62 ± 1.10 (8)

Hepatocytes were incubated for 90 min as described in Methods. Endogenous glucose synthesis was subtracted from values of glucose production from exogenous substrates. The results are mean ± SEM from the number of experiments given in parenthesis.

[a]difference between young and old rats is statistically significant: $p < 0.05$

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gated, both in young and old rats. However, in hepatocytes isolated from young rats the rate of glucagon-stimulated glucose synthesis was much greater than in hepatocytes from old animals when lactate/pyruvate or alanine were the substrates employed. No difference in the rate of glucose synthesis was observed between the two groups of animals in the presence of glucagon when glycerol or fructose were the substrates for glucose production.

Epinephrine and norepinephrine (Table 6) stimulated glucose synthesis from all substrates investigated to the same extent in hepatocytes isolated from both groups of rats compared.

Effect of the exogenous cyclic AMP on glucose synthesis in hepatocytes from young and old rats

It is well established that one of the mechanisms by which glucagon stimulates gluconeogenesis in mammalian liver is an increase in the intracellular concentration of cyclic AMP (Exton, 1968 and 1971). An attempt has been made to mimic glucagon's effect by incubating isolated liver cells with exogenous cAMP. The results of these experiments are shown in Table 5. Both in hepatocytes of young and old rats dibutyryl cAMP was found to mimic well the stimulatory effect of glucagon on glucose synthesis from lactate/pyruvate and from fructose. The effects of glucagon and db cAMP were not additive. In accordance with the results of previous experiments, stimulation of glucose synthesis by db cAMP was significantly greater in hepatocytes of young rats than old ones when lactate/pyruvate but not fructose were the substrates for glucose production.

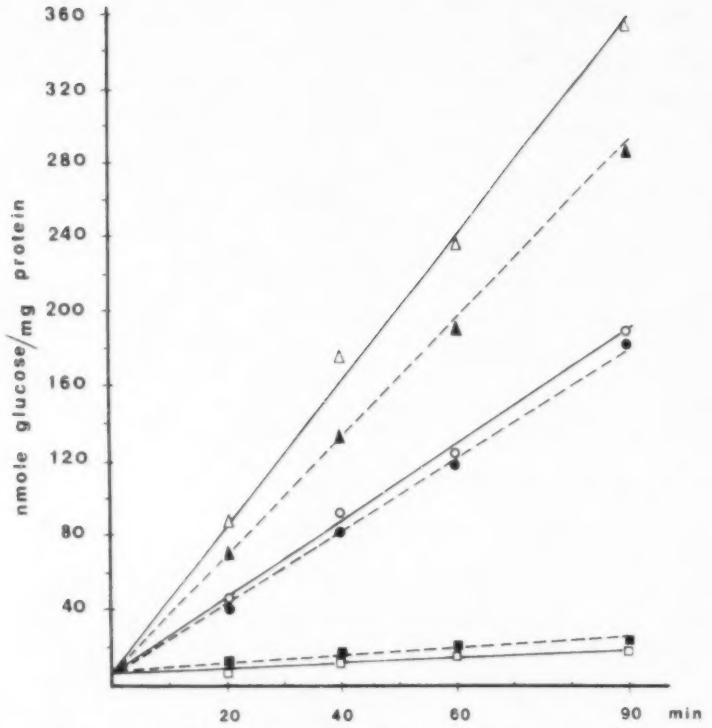

FIG. 3. Time-dependence and the effect of glucagon on glucose production from lactate/pyruvate (18:2 mM) in isolated hepatocytes of young (open symbols) and old (closed symbols) rats. Squares = control, no substrates and hormones added, circles = lactate/pyruvate (18:2 mM), triangles = lactate/pyruvate (18:2 mM) plus glucagon (5×10^{-8}M). Each point represents the mean of 8–10 experiments. Abscissa—time of incubation. Ordinate—production of glucose.

Adrenergic mechanisms of the effect of catecholamines on glucose synthesis in hepatocytes from young and old rats

It is now well established that catecholamines stimulate gluconeogenesis in hepatocytes of rat liver through alpha- and not beta-adrenergic receptors (Tolbert *et al.*, 1973 and 1974; Garrison *et al.*, 1979). However, to our knowledge, the possible influence of aging on these mechanisms of stimulation by catecholamine was not investigated. Therefore, we investigated the effect of propranolol (beta antagonist) and of phenoxybenzamine and dihydroergotamine (both alpha antagonists) on catecholamine-stimulated glucose production in hepatocytes isolated from young and old rats. Data presented in Table 6 indicate that all the adrenolytic agents used did not per se affect the gluconeogenetic function of isolated hepatocytes. In hepatocytes isolated from both age groups, the stimulatory effect of epinephrine and norepinephrine on gluconeogenesis from lactate/pyruvate was completely abolished in the presence of alpha- but not beta-adrenolytic agents. Additionally, isoproterenol, a beta-adrenergic agonist, did not stimulate glucose synthesis in hepatocytes of young and old rats. Therefore it seems possible to conclude that in both age groups studied, catecholamines stimulated gluconeogenesis in rat hepatocytes through the activation of alpha-adrenergic receptors.

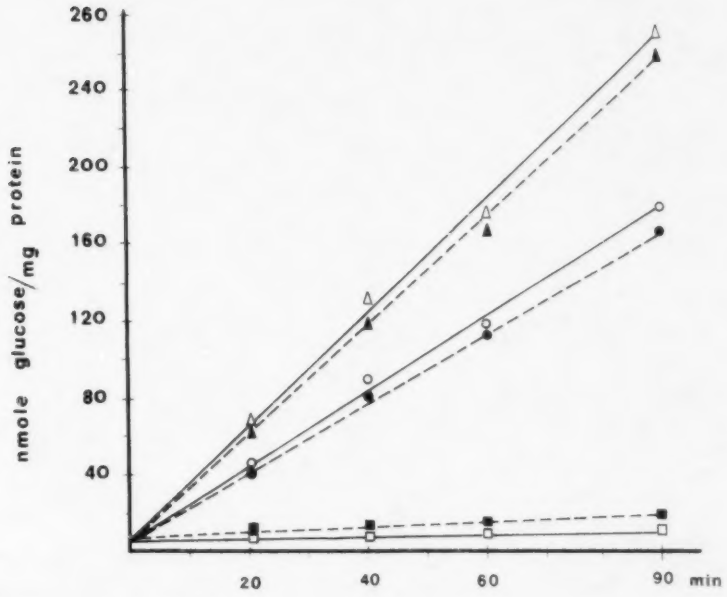

FIG. 4. Time-dependence and the effect of epinephrine on glucose production from lactate/pyruvate (18:2 mM) in isolated hepatocytes of young (open symbols) and old (closed symbols) rats. Squares = control, no substrates and hormones added, circles = lactate/pyruvate (18:2 mM), triangles = lactate/pyruvate (18:2 mM) plus epinephrine (5×10^{-6}M). Each point represents the mean of 7–9 experiments. Abscissa—time of incubation. Ordinate—production of glucose.

DISCUSSION

On the basis of experiments performed the following conclusions can be drawn:

1) In the absence of hormonal stimulation the rate of glucose synthesis from each substrate tested was the same in hepatocytes from both young and old rats.
2) Stimulatory effect of glucagon with lactate/pyruvate or alanine as glucogenic

TABLE 5. EFFECT OF GLUCAGON AND DIBUTYRYL CYCLIC AMP ON GLUCOSE SYNTHESIS FROM LACTATE/PYRUVATE AND FRUCTOSE IN ISOLATED HEPATOCYTES FROM YOUNG AND OLD RATS.

Substrates and stimulators	nmoles glucose/min/mg protein	
	Young rats (6–8 months)	Old rats (20–24 months)
Lactate/pyruvate 18:2 mM	2.05 ± 0.28 (6)	1.88 ± 0.30 (7)
+ glucagon 5×10^{-6}M	4.38 ± 0.48 (6)	3.38 ± 0.40[a] (6)
+ db cAMP 10^{-4}M	4.05 ± 0.48 (5)	3.18 ± 0.35[a] (5)
+ glucagon 5×10^{-6}M + db cAMP 10^{-4}M	4.20 ± 0.38 (5)	3.13 ± 0.43[a] (5)
Fructose 5 mM	5.05 ± 0.58 (5)	4.78 ± 0.53 (6)
+ glucagon 5×10^{-6}M	7.38 ± 0.88 (5)	7.05 ± 0.80 (6)
+ db cAMP 10^{-4}M	7.28 ± 0.83 (4)	7.13 ± 0.83 (4)
+ glucagon 5×10^{-6}M + db cAMP 10^{-4}M	7.10 ± 0.78 (4)	6.98 ± 0.98 (4)

Hepatocytes were incubated for 40 min as described in Methods. The results are mean ± SEM from the number of experiments given in parenthesis. Values given in table were corrected for the endogenous glucose production.

[a]difference between young and old rats is statistically significant: $p < 0.05$.

TABLE 6. EFFECTS OF ADRENERGIC AGONISTS AND ANTAGONISTS ON GLUCOSE SYNTHESIS FROM LACTATE/PYRUVATE (18:2 mM) IN ISOLATED HEPATOCYTES FROM YOUNG AND OLD RATS.

Agents	nmoles/min/mg protein glucose	
	Young rats (6–8 months)	Old rats (20–24 months)
Control—only substrates present	1.65 ± 0.25 (5)	1.49 ± 0.26 (5)
Propranolol 10^{-5}M	1.71 ± 0.30 (4)	1.52 ± 0.27 (4)
Phenoxybenzamine 10^{-5}M	1.62 ± 0.22 (5)	1.51 ± 0.25 (4)
Dihydroergotamine 10^{-5}M	1.75 ± 0.32 (5)	1.45 ± 0.29 (5)
Isoproterenol 5×10^{-6}M	1.53 ± 0.25 (4)	1.45 ± 0.28 (4)
Epinephrine 5×10^{-6}M	2.38 ± 0.33 (5)	2.20 ± 0.28 (6)
+ propranolol 10^{-5}M	2.33 ± 0.30 (5)	2.25 ± 0.33 (5)
+ phenoxybenzamine 10^{-5}M	1.70 ± 0.23 (5)	1.48 ± 0.25 (5)
+ dihydroergotamine 10^{-5}M	1.53 ± 0.23 (5)	1.40 ± 0.20 (5)
Norepinephrine 5×10^{-6}M	2.23 ± 0.30 (5)	2.03 ± 0.28 (5)
+ propranolol 10^{-5}M	2.30 ± 0.28 (5)	1.98 ± 0.25 (5)
+ phenoxybenzamine 10^{-5}M	1.68 ± 0.28 (5)	1.40 ± 0.23 (5)
+ dihydroergotamine 10^{-5}M	1.58 ± 0.23 (5)	1.43 ± 0.25 (5)

Hepatocytes were incubated for 10 min with adrenolytic agents/propranolol, phenoxybenzamine, dihydroergotamine/and then epinephrine or norepinephrine were added and incubation was continued for 40 min. The results are mean ± SEM from the number of experiments given in parenthesis. Values given in table were corrected for the endogenous glucose production.

substrates was less pronounced in hepatocytes from old rats than the young ones, while the stimulation by catecholamines was equally effective in both compared groups of animals.

These conclusions are in accord with the general view that in mammals the basic level of function of a given organ does not change substantially during aging. However, the age-dependent differences of organ function can be unrevealed under the conditions of maximal stimulation (Finch, 1977; Sanadi, 1977; Adelman *et al.*, 1978). The results of experiments presented in this paper indicate that the stimulation need not necessarily reveal age-dependent changes in a given organ. Differences in the rate of glucagon-stimulated glucose synthesis were observed only when hepatocytes were incubated with lactate/pyruvate or alanine as glucogenic substrates. Since the stimulation with glucagon did not reveal differences between the two animal groups if glycerol or fructose were employed as substrates for glucose synthesis, one can suppose that there was no change in the very first stage of glucagon action i.e., activation of adenyl cyclase and the increase of intracellular cAMP level. The differences in glucose synthesis from lactate/pyruvate or alanine in hepatocytes from young and old rats could have been also observed when stimulation of gluconeogenesis was produced by exogenous db cAMP (Table 5). This may indicate that the age-dependent process concerns rather the glucogenic pathway itself and most probably those steps which are involved in the conversion of pyruvate, lactate or alanine, but not glycerol or fructose. Closer look at the sequence of enzymatic reactions of gluconeogenic pathway from various substrates points to those reactions which occur exclusively in mitochondria, namely carboxylation of pyruvate catalysed by pyruvate carboxylase. This is a regulatory enzyme and it has been reported that glucagon and epinephrine activate rat

liver pyruvate carboxylase, the mechanism of this activation being different in case of glucagon and epinephrine (Garrison *et al.*, 1975, 1979).

In our experiments the age-dependent differences were observed only upon stimulation with glucagon. Catecholamine were equally effective in stimulation of gluconeogenesis in both groups of animals and with all glucogenic substrates tested. Therefore, it seems plausible that the decline of glucagon-stimulated glucose synthesis observed in hepatocytes from old rats is caused by a specific, age-dependent change in the properties of pyruvate carboxylase. However, at present there is insufficient direct experimental data supporting this hypothesis.

Isolated hepatocytes of rats and other mammals are, at present, commonly used as a functional equivalent of the whole organ in the intact organism (Seglen, 1975). Therefore, it can be stated that the reported age-dependent decline in the response to glucagon stimulation applies also to the function of the whole liver in old organism. Such impairment of liver function can negatively influence the adaptive ability of the aging organism in the situation of growing requirement for glucose, the main energetic substrate for brain and other tissues (Exton, 1972). It has been found that after overnight fast the human liver produces 10 g of glucose per hour which is equal to the whole amount of extracellular pool of glucose, and 75% of that amount is produced as a result of glucagon stimulation (Cherrington *et al.*, 1981). During the first days of fasting the liver is the only source of glucose necessary for maintaining functions of the organism (Exton, 1972). In such situations in old organisms the impairments of gluconeogenic response of liver can decrease their ability to maintain homeostasis of glucose. Findings reported in this paper indicate the importance of the research concerning the metabolism of aging organisms especially under the conditions of hormonal stimulation.

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EFFECT OF CENTROPHENOXINE ON THE ANTIOXIDATIVE ENZYMES IN VARIOUS REGIONS OF THE AGING RAT BRAIN

D. Roy, D.N. Pathak, R. Singh*

Neurobiology Unit, School of Life Sciences, Jawaharlal Nehru University, New Delhi – 110067, India.

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Abstract – This study investigated the effect (*in vivo*) of centrophenoxine (Helfergin) on the activity of antioxidant enzymes (glutathione peroxidase GSH-PER, glutathione reductase GSSG-RED, superoxide dismutase SOD and catalase) in subcellular fractions from the regions of the brain (cerebrum, cerebellum and brain stem) of rats aged 6, 9 and 12 months. In all age groups, normal (control) activity of GSH-PER, GSSG-RED and SOD in the three brain regions was higher in the soluble fractions than in the particulate fractions. The three regions of the brain showed different levels of the enzyme activities. Enzymes in soluble fractions (except GSSD-RED in cerebrum of rats aged 12 months) did not change with age. In particulate fractions, however, the enzymes showed age-related changes: GSH-PER decreased with age in cerebellum and brain stem, but showed an age-related increase in cerebrum, GSSG-RED and SOD increased with age in all the three brain regions. Catalase activity in all the three brain regions remained unchanged in all age groups.

Six week administration of centrophenoxine (once a day in doses of 80 mg/Kg and 120 mg/Kg) to the experimental animals produced increases in the activity of SOD, GSH-PER and GSSG-RED in particulate fractions from all the three brain regions. In the soluble fractions, however, only SOD and GSH-PER activity was increased. *In vitro* also centrophenoxine stimulated the activity of GSH-PER. A dosage of 80 mg/Kg produced greater changes than a 120 mg/Kg dosage. The drug had no effect on the activity of catalase. Centrophenoxine also reduced lipofuscin deposits (studied both biochemically and histochemically) thus indicating that the drug inhibited lipofuscin accumulation by elevating the activity of the antioxidant enzymes. The data suggest that alleviation of senescence by centrophenoxine may, at least, partly be due to activation by it of antioxidant enzymes.

INTRODUCTION

Increasing free radical-induced peroxidation of membrane lipids occurring with age in the body tissue is thought to contribute significantly to age-related impairment of structure and function (Bondareff, 1964; Tappel *et al.*, 1973; Armstrong, 1974; Nohal and Hegner, 1978; Sinet *et al.*, 1980). The peroxidative damage in membranes can result from the action of several oxidants such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxide radical (.OH) which arise from reduction of oxygen. Antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) constitute a natural defense system against the activity of oxidants. An important cytological index of the peroxidative

*To whom all correspondence should be addressed.

damage (lipid-peroxidation) is the accumulation in the nerve cells of lipofuscin popularly known as age-pigment (Singh and Mukherjee, 1972; Davison, 1980) which is a copolymer of proteins and peroxidized lipids (Taubold, 1975). The accumulation of lipofuscin seems to be integratively proportional to the occurrence of lipid peroxidation (Chance *et al.*, 1979).

Centrophenoxine (an ester of dimethylaminoethanol and chlorophenoxy acetic acid) is an antiaging drug, sometimes used for psychogeriatric treatment of various disorders in elderly patients (Nandy, 1978; Olpe and Steinmann, 1981). This drug has been found to stimulate brain metabolism, increase lifespan, improve learning capacity (Nandy, 1978; Hochschild, 1973; Zs-Nagy *et al.*, 1979). The drug has also been shown to produce beneficial effects in some human pathological states such as cerebral atrophy, brain injury, chronic alcoholism (Charbaut *et al.*, 1973; Desterm, 1961; Thuiller *et al.*, 1959; Gustafson *et al.*, 1978). The biochemical mechanism(s) of centrophenoxine action, however, has not been clearly elucidated (Jamieson and Taylor, 1981). Gerontologically, it is of interest that in experimental animals centrophenoxine reduces the formation of lipofuscin (a peroxidation product) suggesting thereby that the drug might possibly act, in some way, interfering with the reactions that promote peroxidation. Reduction in the lipofuscin has sometimes also been found to be associated with increases in lifespan of the organism (Hochschild, 1973). One possibility that merits consideration is whether the drug modulates the activity of antioxidant enzymes which offer varying degrees of protection against cytotoxic action of oxidants. The objective of the present work, thus was to determine the effect of centrophenoxine on some antioxidant enzymes. This was expected to provide answer to the question whether centrophenoxine has the potentiality to produce gerontologically beneficial effects by activating chemical pathways that can retard peroxidation.

In this paper are presented the data on the effects of centrophenoxine on the activity of antioxidant enzymes: (1) superoxidase dimutase (EC 1.15.1.1.), catalase (EC 1.11.1.6), (2) glutathione peroxidase (EC 1.11.1.6) and (3) glutathione reductase (EC 1.6.4.2) in the various regions of the brain of rats of 6, 9 and 12 months of age.

METHODS AND PROCEDURE

Female Albino rats of the Holtzman strain of three different ages viz. 6, 9 and 12 months were used in this study. Each age-group was subdivided into three sets: one set served as control, the second received 80 mg/kg dose of the drug and the third received 120 mg/kg dose of the drug, centrophenoxine.

Each dose of the drug (dissolved in physiological saline) was administered by intraperitoneal injection once a day for 6 weeks. Control animals were injected with same volume of saline. All three groups were given food and water ad libitum.

Rats were killed by cervical dislocation, brain was quickly dissected out and cerebral hemispheres, cerebellum and brain stem were excised immediately and weighed. Tissue homogenates (1:10) were prepared in ice-cold 0.25 M sucrose. Differential centrifugation was performed according to the methods of Mayanil *et al.* (1982). Fractions were obtained at 17,000 g pellets were washed with homogenizing medium. The pellet containing mitochondrial-synaptosomal fraction and soluble fraction or supernatant were used separately for all enzyme activity assays. Superoxide dismutase (SOD) was assayed according to the method of Thomas *et al.* (1976), catalase (C-ase) was assayed in post nuclear supernatant (PNS) separated at 1000 g according to the method of Aebi (1974); glutathione peroxidase (GSH-PER) was measured by the method of Prohaska and Ganther (1976) and Glutathione reductase (GSSG-RED) was determined by the procedure of Chance *et al.* (1979). To determine whether centrophenoxine administration in rats resulted in lipofuscin reduction, lipofuscin content of brain was determined spectrofluorimetrically in experimental and control animals. Method of Tappel *et al.* (1973) was used for biochemical estimation of lipofuscin. Influence of centrophenoxine administration was also assessed in histochemically stained paraffin sections of the brain of both experimental and control animals. Histochemical

methods used were: Cresyl violet (Samorajski *et al.*, 1965), Paraldehyde fuchsin (Singh and Mukherjee, 1972), autofluorescence (Nandy, 1971).

Statistical analysis

The data were analysed using students '*t*' test.

RESULTS

Normal activity levels of antioxidant enzymes in the brain

Values of the activity levels of the four enzymes viz. Glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase estimated in controls of various age-groups are given in Tables 1–3. Results show that the three enzymes, SOD, GSH-PER and GSSG-RED were detectable both in cytosol and crude mitochondrial fractions from all the three regions of the brain. Significant levels of catalase activity were detected in the postnuclear fractions from all the three regions of brain. The data further showed that mostly higher activity levels of enzymes occurred in cytosol fractions (soluble fraction) as compared to those in the mitochondrial fractions (Tables 1–3).

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In all the three age groups (6, 9 and 12 months) the enzymes in the cytosol fractions from all the three regions of brain mostly did not show any age-related change in their activity whereas the enzymes in the particulate fractions from all the three brain regions did show age-related changes (Tables 1–3). GSH-PER activity decreased with age both in cerebellum and brain stem, but it showed an age-related increase in cerebral hemispheres. The other two enzymes, SOD and GSSG-RED showed increases with age in all the three brain regions. In the case of cytosol fractions, only in cerebral hemisphere GSSG-RED activity showed an increase in the 12 months age group. Of the three regions of the brain, maximum level of activity of GSH-PER and GSSG-RED occurred in brain stem. The cerebral hemisphere and cerebellum showed less enzyme activity (brain stem > cerebral hemisphere > cerebellum). The SOD and catalase activities also were found to be maximum in brain stem followed by cerebellum and cerebral hemispheres (brain stem > cerebellum > cerebral hemispheres).

Effect of centrophenoxine on catalase activity

Both doses of centrophenoxine (80 mg and 120 mg) produced no changes in the activity of catalase in any of the three regions of the brain.

Effect of centrophenoxine on superoxide dismutase activity

Whereas 120 mg/kg dose of centrophenoxine produced less increases in the activity of SOD in cytosol fraction from all the three regions of brain of all the three age groups, the 80 mg/kg dose, however, produced more significant increases in the enzyme activity (Fig. 1). In the mitochondrial fractions both doses of the drug produced significant increases in SOD activity. However, increases in the enzyme activity produced by 80 mg dose were more than that produced by 120 mg dose in all the three regions of the brain of all the three age groups. In mitochondrial fraction maximum increase in SOD activity was seen with 80 mg/kg dose in cerebrum followed by cerebellum and brain stem (Fig. 2).

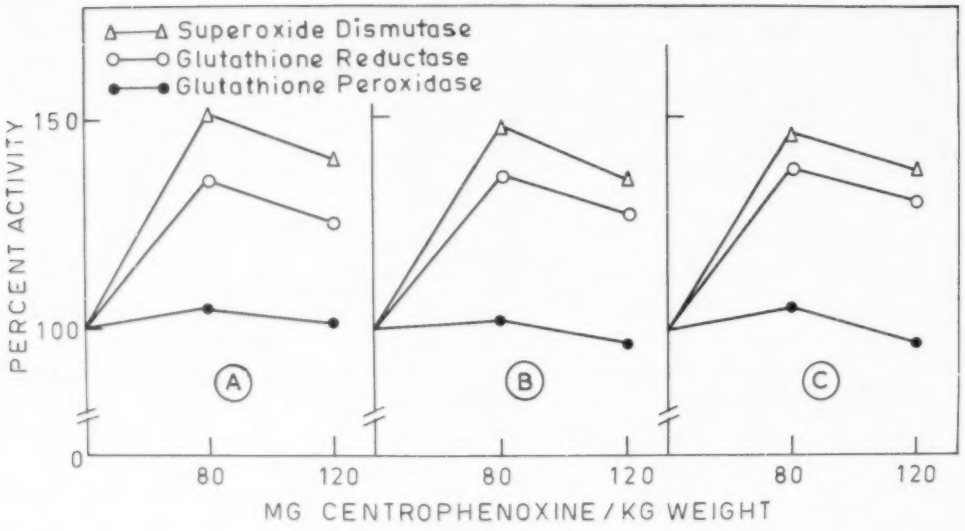

FIG. 1. Effect of centrophenoxine on the activity of antioxidant enzymes shown as percentage change in cytosol (soluble fraction) of various regions of brain of 12 months-old rat. (Control taken as 100%). (A) Cerebral hemisphere, (B) Cerebellum, (C) Brain stem.

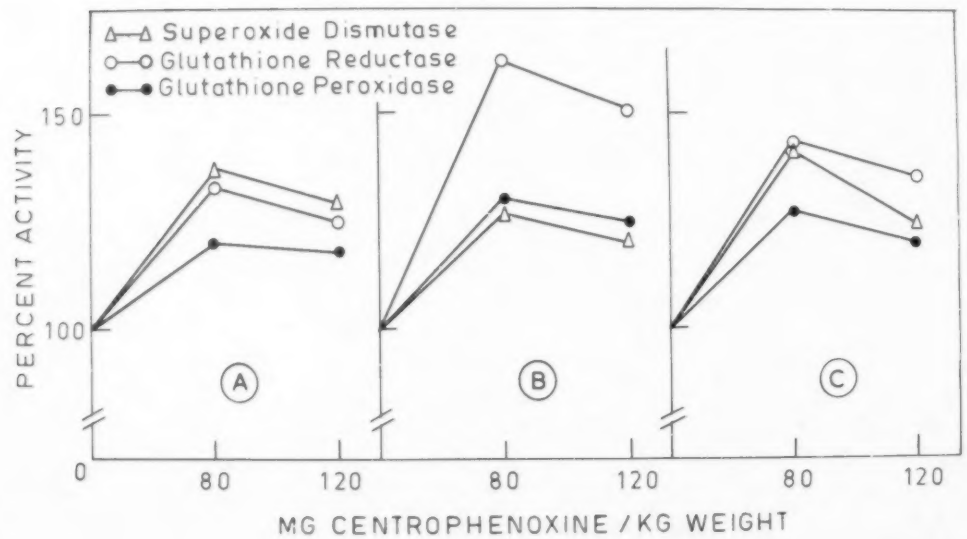

FIG. 2. Effect of centrophenoxine on the activity of antioxidant enzymes shown as percentage change in pellet at 17,000 g (mitochondrial fraction) of various regions of brain of 12 months-old rat. (Control activity taken as 100%). (A) Cerebral hemisphere, (B) Cerebellum, and (C) Brain stem.

TABLE 1. EFFECT OF CENTROPHENOXINE ON ANTIOXIDANT ENZYMES IN CEREBRAL HEMISPHERE OF VARIOUS AGE-GROUPS.

Enzymes studied	Crude mitochondrial fraction at 17,000 g			Soluble fraction at 17,000 g		
		Treated			Treated	
	Control	80 mg/kg	120 mg/kg	Control	80 mg/kg	120 mg/kg
6 months old:						
GSH-PER	0.730 ± 0.010	0.980 ± 0.030*	0.960 ± 0.040**	1.500 ± 0.120	1.630 ± 0.090	1.450 ± 0.200
GSSG-RED	0.450 ± 0.010	0.660 ± 0.050**	0.580 ± 0.023*	0.880 ± 0.170	1.110 ± 0.040**	1.060 ± 0.060***
SOD	0.260 ± 0.015	0.360 ± 0.025**	0.400 ± 0.012*	0.600 ± 0.025	0.900 ± 0.035*	0.820 ± 0.030**
C-ase†				0.120 ± 0.005	0.125 ± 0.001	0.122 ± 0.007
9 months old:						
GSH-PER	0.930 ± 0.080	1.220 ± 0.005**	1.170 ± 0.020*	1.650 ± 0.160	1.400 ± 0.300	1.600 ± 0.120
GSSG-RED	0.640 ± 0.010	0.890 ± 0.006*	0.840 ± 0.002*	1.050 ± 0.120	1.290 ± 0.040*	1.250 ± 0.026
SOD	0.380 ± 0.040	0.480 ± 0.010**	0.500 ± 0.003**	0.680 ± 0.008	1.000 ± 0.006*	0.890 ± 0.040**
C-ase†				0.136 ± 0.002	0.124 ± 0.008	0.135 ± 0.010
12 months old:						
GSH-PER	0.840 ± 0.003	1.010 ± 0.004***	0.990 ± 0.009****	1.540 ± 0.150	1.450 ± 0.200	1.580 ± 0.300
GSSG-RED	0.780 ± 0.006	1.040 ± 0.040**	0.987 ± 0.010**	1.160 ± 0.240	1.560 ± 0.040*	1.450 ± 0.450*
SOD	0.520 ± 0.004	0.700 ± 0.025*	0.660 ± 0.004**	0.660 ± 0.060	1.000 ± 0.009**	0.960 ± 0.006***
C-ase†				0.125 ± 0.002	0.120 ± 0.004	0.118 ± 0.006

Each value is mean ± S.E.M. of six or more experiments.

†Catalase (C-ase) activity was assayed in post nuclear supernatant PNS.

Asterisk marked values are only significant: *$p < 0.01$ **$p < 0.02$ ***$p < 0.03$ ****$p < 0.04$.

Values of GSH-PER & GSSG-RED are given in μ moles NADPH oxidized/gm tissue/min., whereas C-ase & SOD are expressed in Units/gm tissue.

TABLE 2. EFFECT OF CENTROPHENOXINE ON ANTIOXIDANT ENZYMES IN CEREBELLUM OF VARIOUS AGE-GROUPS.

Enzymes studied	Crude mitochondrial fraction at 17,000 g			Soluble fraction at 17,000 g		
		Treated			Treated	
	Control	80 mg/kg	120 mg/kg	Control	80 mg/kg	120 mg/kg
6 months old:						
GSH-PER	0.790 ± 0.008	1.050 ± 0.010**	0.950 ± 0.035*	1.600 ± 0.300	1.480 ± 0.260	1.550 ± 0.240
GSSG-RED	0.190 ± 0.002	0.410 ± 0.070*	0.370 ± 0.020*	0.740 ± 0.060	0.990 ± 0.040**	0.910 ± 0.030**
SOD	0.420 ± 0.020	0.580 ± 0.040*	0.540 ± 0.012	0.750 ± 0.056	1.200 ± 0.018**	1.090 ± 0.040***
C-ase†				0.340 ± 0.120	0.350 ± 0.026	0.345 ± 0.028
9 months old:						
GSH-PER	0.680 ± 0.016	0.860 ± 0.020**	0.820 ± 0.013	1.400 ± 0.150	1.450 ± 0.120	1.520 ± 0.200
GSSG-RED	0.290 ± 0.060	0.500 ± 0.017*	0.450 ± 0.010*	0.660 ± 0.040	0.800 ± 0.040***	0.760 ± 0.020
SOD	0.360 ± 0.018	0.540 ± 0.008*	0.480 ± 0.030**	0.720 ± 0.030	1.120 ± 0.008**	1.020 ± 0.040****
C-ase†				0.348 ± 0.042	0.345 ± 0.026	0.355 ± 0.092
12 months old:						
GSH-PER	0.520 ± 0.012	0.690 ± 0.016**	0.650 ± 0.011***	1.440 ± 0.240	1.550 ± 0.160	1.360 ± 0.120
GSSG-RED	0.400 ± 0.025	0.650 ± 0.030*	0.600 ± 0.020**	0.750 ± 0.050	1.020 ± 0.020*	0.950 ± 0.010**
SOD	0.580 ± 0.010	0.740 ± 0.025*	0.700 ± 0.010*	0.760 ± 0.040	1.120 ± 0.060*	1.030 ± 0.010*
C-ase†				0.342 ± 0.012	0.347 ± 0.006	0.336 ± 0.014

Each value is mean ± S.E.M. of six or more experiments.

†Catalase activity (C-ase) was assayed in PNS.

Asterisk marked values are only significant: *$p < 0.01$ **$p < 0.02$ ***$p < 0.03$ ****$p < 0.04$.

Values of GSH-PER & GSSG-RED are given in μ moles NADPH oxidized/gm tissue/min., whereas C-ase & SOD are expressed in Units/gm tissue.

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TABLE 3. EFFECT OF CENTROPHENOXINE ON ANTIOXIDANT ENZYMES IN BRAIN STEM OF VARIOUS AGE-GROUPS.

Enzymes studied	Crude mitochondrial fraction at 17,000 g			Soluble fraction at 17,000 g		
		Treated			Treated	
	Control	80 mg/kg	120 mg/kg	Control	80 mg/kg	120 mg/kg
6 months old:						
GSH-PER	1.120 ± 0.060	1.450 ± 0.040**	1.360 ± 0.060****	2.120 ± 0.160	2.040 ± 0.040	1.990 ± 0.090
GSSG-RED	0.600 ± 0.018	0.850 ± 0.045*	0.780 ± 0.003**	1.660 ± 0.190	2.180 ± 0.026**	2.020 ± 0.040***
SOD	0.420 ± 0.020	0.580 ± 0.008*	0.530 ± 0.001	0.920 ± 0.008	1.200 ± 0.010*	1.080 ± 0.020
C-ase†				0.375 ± 0.002	0.370 ± 0.025	0.373 ± 0.020
9 months old:						
GSH-PER	1.010 ± 0.020	1.280 ± 0.040***	1.260 ± 0.010***	1.960 ± 0.040	2.050 ± 0.017	1.980 ± 0.020
GSSG-RED	0.750 ± 0.010	1.050 ± 0.035**	0.980 ± 0.050***	1.560 ± 0.050	1.960 ± 0.006**	1.840 ± 0.040***
SOD	0.500 ± 0.008	0.650 ± 0.005*	0.610 ± 0.003**	0.880 ± 0.006	1.300 ± 0.070*	1.140 ± 0.008***
C-ase†				0.368 ± 0.020	0.373 ± 0.009	0.365 ± 0.026
12 months old:						
GSH-PER	0.900 ± 0.002	1.140 ± 0.006**	1.080 ± 0.004***	2.020 ± 0.020	2.100 ± 0.015	1.950 ± 0.030
GSSG-RED	0.830 ± 0.060	1.200 ± 0.030*	1.120 ± 0.020**	1.600 ± 0.010	2.200 ± 0.019*	2.080 ± 0.090**
SOD	0.450 ± 0.002	0.970 ± 0.009*	0.810 ± 0.009*	0.980 ± 0.006	1.430 ± 0.060*	1.340 ± 0.020**
C-ase†				0.370 ± 0.012	0.363 ± 0.016	0.375 ± 0.010

Each value is mean ± S.E.M. of six or more experiments.

†Catalase activity (C-ase) was assayed in PNS.

Asterisk marked values are only significant: *$p < 0.01$ **$p < 0.02$ ***$p < 0.03$ ****$p < 0.04$.

Values of GSH-PER & GSSG-RED are given in μ moles NADPH oxidized/gm tissue/min., whereas C-ase & SOD are expressed in Units/gm tissue.

Effect of centrophenoxine on glutathione peroxidase activity

Both the doses of centrophenoxine produced no changes in GSH-PER activity in cytosol fractions from all the three regions of brains of all the three age groups. In mitochondrial fraction, however, significant increases in GSH-PER activity were seen with both doses of the drug. Maximum increase was observed in cerebellum followed by brain stem and cerebrum. Quantitatively increases produced by 80 mg dose were higher than those produced by 120 mg dose (Fig. 1; Tables 1–3).

Effect of centrophenoxine on glutathione reductase activity

Both the doses of the drug produced increases in GSSG-RED activity in both fractions (mitochondrial and cytosol) from all the three brain regions. Increases produced by 80 mg dose were higher than those by 120 mg. In mitochondrial fraction, centrophenoxine produced greatest increase in GSSG-RED activity in cerebellum followed by brain stem and cerebrum. In cytosol fraction, however, maximum increase was produced in brain stem followed by cerebrum and cerebellum.

In conclusion the data showed that whereas catalase activity was not influenced by centrophenoxine, the activity of the other three enzymes SOD, GSH-PER and GSSG-RED was increased by centrophenoxine in all the three brain regions in all the three age groups. However, the enzymes in two subcellular fractions sometimes differed in their response to centrophenoxine, for instance in mitochondrial fractions all the three enzymes (SOD, GSH-PER and GSSG-RED) showed an increase with both doses of centorphenoxine, but glutathione peroxidase activity in cytosol fractions was not influenced by any of the two doses. Further, a 80 mg dose always produced enzyme activity increases which were larger than those produced by a 120 mg dose. The present data would thus show that centrophenoxine significantly increases the activity of three antioxidant enzymes; SOD, GSH-PER and GSSG-RED in all the three regions of brain of the age groups (6, 9 and 12 months) examined in the present work.

Effect of centrophenoxine in vitro on glutathione peroxidase activity

Experiments were also performed to find out whether centrophenoxine *in vitro* increased the activity of GSH-PER. The data presented in Table 4 show that centrophenoxine significantly increases the GSH-PER activity *in vitro* also. GSH-PER plays actually a major antioxidant role beause it constitutes a repair mechanism by which it can detoxify peroxidized lipid membranes by directly interacting with their peroxidated lipids. It can also detoxify H_2O_2 to H_2O (Chance *et al.*, 1979). It has been reported that GSH-PER may be more relevant for protection against H_2O_2 than catalase (Bartosz and Bartkowiak, 1981). Therefore, the *in vitro* observation made in the present study that centrophenoxine increases GSH-PER activity together with the *in vivo* observation showing the same, shows that centrophenoxine is likely to activate natural antioxidant defense mechanisms to protect tissues against oxidant-caused damage.

Effect of centrophenoxine on flourescent products (lipofuscin)

Comparison of histological preparations from drug treated animals with those from controls, showed that the former had reduced deposits of lipofuscin in their cells (Fig. 3). These observations were generally similar to those already published in many studies (Riga nd Riga, 1974; Nandy, 1968, 1978). Biochemical data (Table 5) from experimental and control preparation showed reduced lipofuscin content in drug treated animals.

TABLE 4. IN VITRO STUDIES OF EFFECT OF CENTROPHENOXINE ON GLUTATHIONE PEROXIDASE IN CEREBRAL HEMISPHERE.

	GSH-PER Activity μ moles/gm/min	% Change
Control	1.03 ± 0.05	100%
treated		
10^{-2}M	1.42 ± 0.08*	138%
10^{-3}M	1.60 ± 0.02*	156%
10^{-4}M	1.36 ± 0.04**	134%
10^{-5}M	1.25 ± 0.009**	125%
10^{-6}M	1.21 ± 0.006***	120%

Whole homogenate was incubated at 27°C along with centrophenoxine for 75 minutes and activities were assayed.

Each value is a mean ± SEM of four or more experiments.

Control taken as 100%.

Asterisks indicate statistical significance: *$p < .01$, **$p < .02$, ***$p < .03$.

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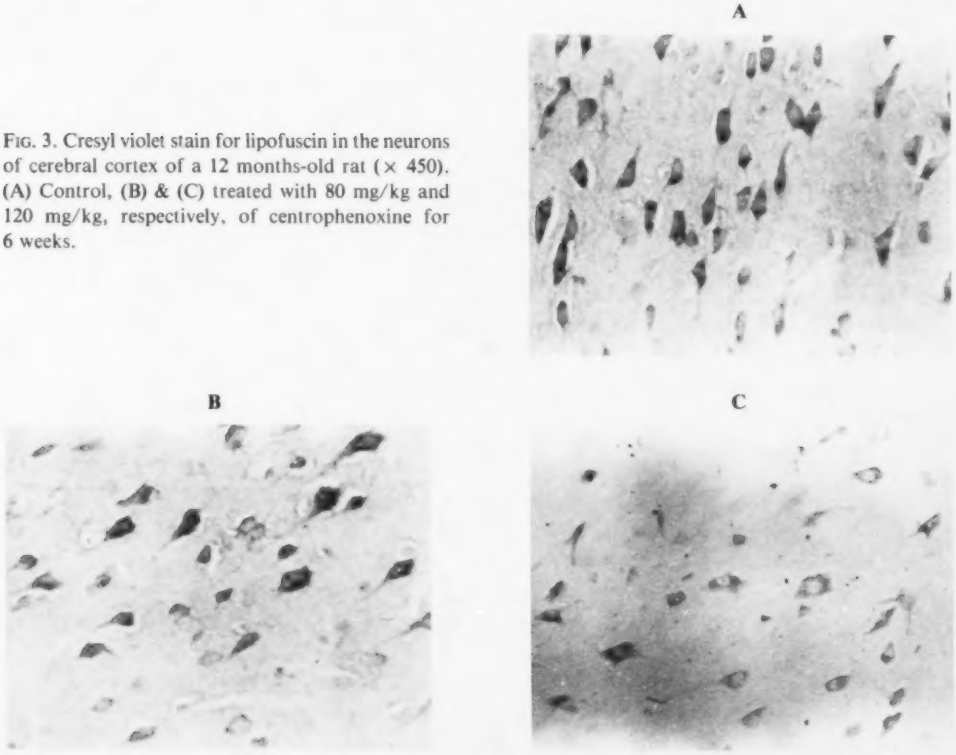

FIG. 3. Cresyl violet stain for lipofuscin in the neurons of cerebral cortex of a 12 months-old rat (× 450). (A) Control, (B) & (C) treated with 80 mg/kg and 120 mg/kg, respectively, of centrophenoxine for 6 weeks.

TABLE 5. EFFECT OF CENTROPHENOXINE ON FLUORESCENT PRODUCTS FOR 0.2 GM OF BRAIN TISSUES OF RAT OF DIFFERENT AGE GROUPS.

Age in months	Control Fluorescence units ± SEM	Treated	
		80 mg/kg	120 mg/kg
6	80.27 ± 4.60	60.05 ± 5.09**	52.45 ± 9.33*
9	95.79 ± 6.92	69.92 ± 8.9**	67.92 ± 6.9*
12	120.63 ± 11.02	78.12 ± 3.26*	65.43 ± 15.19*

Each value is a mean ± SEM of four or more experiments.
Values with asterisk marks are significantly different from controls: $*p < .01$, $**p < .02$.

DISCUSSION

Data obtained in the present work showed that SOD, GSH-PER and GSSG-RED activities occurring in soluble fractions or cytosol fractions from different regions of the brain of various age-groups were generally higher than that occurring in corresponding mitochondrial fractions. This seems to be in general agreement with previous reports (Prohaska and Ganther, 1976; Chance *et al.*, 1979; Thomas *et al.*, 1976; Brannan *et al.*, 1980), on the distribution of antioxidant enzyme activities between cytosol and particulate fractions from various tissues. In these reports it was observed that more antioxidant enzyme activity was localized in cytosol fraction than in particulate fraction.

Another interesting finding which emerged from the present work is that cytosol antioxidant enzymes were different from particulate fraction enzymes in their response to age. For example, cytosol enzymes did not show any change in their activity with age, whereas particulate fraction enzymes showed an age-related increase or decrease. Mitochondrial SOD and GSSG-RED increased with age in all the three regions of brain, GSH-PER decreased with age in cerebellum and brain stem, but it showed an age-related increase in cerebral hemisphere. In cytosol, only GSSG-RED showed some increase with age. Previous studies have used only the whole homogenates (Mavelli *et al.*, 1978; Chance *et al.*, 1979; Hothersal *et al.*, 1981), our study is different as the enzyme activities have been measured in particulate fraction containing mitochondria and synaptosome and the soluble fractions containing cytosol and microsome, separately. Catalase did not show any change with age and regional distribution of Catalase observed in our study was similar to the observation reported by Gaunt and DeDuve (1967). Different regions of the brain showed different levels of activity of various enzymes. The differential activity of these enzymes in different regions may be responsible for different rates of aging in different parts of the brain.

Concerning the effect of centrophenoxine on antioxidant enzymes, the present results indicated that whereas the catalase activity is not affected by the drug, activity of all the other three enzymes (SOD, GSH-PER and GSSG-RED), is influenced by centrophenoxine. Further there were some differences in the response to centriophenoxine of the antioxidant enzymes of cytosol and those of mitochondrial fractions. For instance GSH-PER in cytosol was not influenced by both doses of the drug used in this investigation whereas the mitochondrial fractions GSH-PER was stimulated by the drug. Since the nature of the enzymes localized at mitochondrial site is different from that of the enzymes localized in

cytosol (Chance *et al.*, 1979), the difference in responses to the drug may probably be due to the differences in the nature of enzymes at the two sites.

Centrophenoxine stimulated the SOD activity in all the three regions of brain. It would thus appear that centrophenoxine can enhance the dismutation of superoxide anion and, hence, reduce the damage likely to result from the activity of superoxide anion. Dismutation of O_2^- radical, however, would result in the increased formation of H_2O_2. Since Catalase activity was not enhanced by centrophenoxine, the elevation of H_2O_2 might favor increased H_2O_2-induced lipid peroxidation if normal activity level of Catalase fails to decompose H_2O_2. That is why probably in some *in vitro* studies (Jamieson and Taylor, 1981), centrophenoxine was not found to prevent initial lipid peroxidation. However, to a certain extent glutathione system can also detoxify H_2O_2 (Chance *et al.*, 1979). The present results showed that centrophenoxine *in vivo* enhances the activities of both the enzymes of glutathione system, i.e., GSH-PER and GSSG-RED. GSH-PER is thought to limit lipid peroxidation initiated by oxidants, this enzyme can further act on peroxidated lipids to restore them to lipid alcohol, and it has been generally accepted that glutathione dependent enzymes, particularly GSH-PER, decompose long chain lipid peroxide (Chance *et al.* 1979). GSH-PER can, therefore, offer sufficient protection against lipid peroxidation. Centrophenoxine's induction of GSH-PER activity thus further testify to the centrophenoxine's potential for limiting the lipid peroxidation. The present data also showed that GSSG-RED activity is stimulated by centrophenoxine treatment. The GSSG-RED activity increased observed in the present work may be attributable to increased availability of reduced glutathione a substrate for GSH-PER.

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McCay *et al.* (1976) reported that GSH-PER inhibited lipid peroxidation in biomembranes by preventing free radical attacks on the polyunsaturated lipids rather than by directly reducing lipid-perioxides. However, Gibson *et al.* (1980) could not find evidence in favor of the inhibition of lipid-peroxidation by GSH-PER. Most reports are in favor that glutathione peroxidase plays a role in prevention of lipid peroxidation (Chow and Tappel, 1972; Brain and Siegal, 1980; Chance *et al.*, 1979; Hothersal *et al.*, 1981; Kappus and Sies, 1981), although the underlying mechanism remains unclear. Therefore, whatever may be the mechanism, stimulation of GSH-PER by centrophenoxine is likely to help reduction of the damage resulting from lipid peroxidation.

Since reports from a number of laboratories have shown that centrophenoxine causes reduction in the formation of lipofuscin (Nandy, 1968, 1978; Riga and Riga, 1974; Spoerri and Glees, 1975), which is an indicator of peroxidative damage, it would appear clear that centrophenoxine-induced decrease in lipofuscin may be because of the inhibition of lipid peroxidation which in turn may be brought about by enhanced activity of antioxidant enzymes: GSH-PER, GSSG-RED and SOD. In the present experiments also it was found that lipofuscin accumulation was decreased by centrophenoxine. It would thus seem probable that activity level of the antioxidant enzymes can play an important role in determining the rate at which lipofuscin can be formed. The present results thus provide significant information concerning the mechanism by which lipofuscinolytic agents like centrophenoxine may bring about the reduction in age-pigments (lipofuscin).

It may be further emphasized that centrophenoxine can only reduce the formation of lipofuscin but it cannot prevent its formation altogether (Nandy, 1976, 1978). It would appear that the stimulation of antioxidant enzymes SOD, GSH-PER, GSSG-RED by centrophenoxine may not be sufficient to completely stop lipid peroxidation. Thus, initial

formation of lipid-peroxides can still lead to the formation of lipofuscin. However, increased GSH-PER activity is likely to reduce the availability of lipid peroxides for lipofuscin formation as the former may be decomposed to lipid alcohols or fatty acids. Lipid alcohol or fatty acids formed by the reduction of lipid-peroxides (Christopherson, 1968) can be metabolized via the β-oxidation pathway. Studies of Jamieson and Taylor (1981) attempted to show that centrophenoxine did not prevent lipid peroxidation, but since they measured only the initial lipid-peroxidation, their observations may not justify the conclusion that centrophenoxine cannot suppress lipofuscin formation through prevention of lipid peroxidation.

SUMMARY

The present study investigated the effects of centrophenoxine on antioxidant enzymes in various regions of rat brain of different age-groups (6 months, 9 months and 12 months old). Two groups of rats received 80 mg and 120 mg/kg, respectively of centrophenoxine intraperitoneally for 6 weeks. A third group of rats received saline daily for 6 weeks, serving as control for both dosage levels. Treatment with centrophenoxine (both doses) resulted in significant increases in the activity of GSH-PER, GSSG-RED, and SOD in mitochondrial fraction from various regions of brain in all age groups. In cytosol there was increase in the level of activity of GSSG-RED and SOD in various regions of brain of all the three age-groups whereas there was no change in the activity of GSH-PER. Catalase activity did not show any change following treatment with centrophenoxine. The data obtained suggest that centrophenoxine may reduce lipofuscin by influencing the activity of antioxidant enzymes.

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AGE-RELATED CHANGES IN THERMOREGULATION IN MALE ALBINO RATS

T. BALMAGIYA* and S.J. ROZOVSKI

Institute of Human Nutrition & School of Public Health, Faculty of Medicine, Columbia University, 701 West 168th Street, New York, N.Y. 10032

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Abstract—Male Sprague Dawley rats were followed longitudinally from 3 to 24 months of age. Resting oxygen consumption (VO_2), measured in the thermal neutral zone (29 ± 1.0°C) decreased 47% between 3 and 24 months of age with a stable period from 6 to 9 months. Changes in rectal temperature in general followed changes in VO_2. On the average the decline in rectal temperature from 3 to 24 months was 0.8°C. Thermal conductance dropped initially from 3 to 6 months and remained stable during further age periods. Thermal circulation index rose slightly from 3 to 13 months, and dropped thereafter from 13 to 24 months. When animals were exposed to a mild cold challenge (18–19°C for 90 min.), the increase in VO_2 was the same from 3 to 13 months of age. At 24 months this increase was significantly higher. The capacity for non-shivering thermogenesis (NST) measured after norepinephrine stimultion declined from 3 to 6 months, remained stable from 6 to 9 months and declined to 13 months. The capacity for NST after a mild cold challenge was significantly decreased at 24 months of age. These results suggest that shivering thermogenesis (ST) may be the main source of heat production in the old organism when faced with a mild cold challenge. Since ST is more energy consuming than NST it may explain the accidental hypothermia which occurs often in the elderly.

Indexing key words: aging, thermoregulation, energy metabolism, rats.

INTRODUCTION

THERMAL REGULATION in old age is less efficient than in the young (Shock, 1977; Green, 1981). An age-related decline in autonomic homeostatic thermoregulation is associated with thermal instability and high risk of accidental hypothermia (Fox *et al.*, 1973; Horvath and Rochelle, 1977; Drinkwater and Horvath, 1979; Collins, 1977; MacLean and Emslic-Smith, 1978; Vaughan *et al.*, 1981). Some authors have suggested that disthermia in the elderly is part of a general impairment in the physiological adaptation to changes in the environment during aging (Finch and Hyflick, 1977; Comfort, 1979; Timiras, 1972), mostly due to a decline of the regulatory function of the hypothalamo-pituitary system (Everitt, 1980; Frolkis, 1976). In addition, the threshold sensitivity of the hypothalamus to neurotransmitters has been shown to be elevated in aged animals (Dilman, 1981; Clark and Lipton, 1981). The ability of old humans and animals to resist a cold challenge is

*To whom all correspondence should be addressed.

reduced (Finch *et al.*, 1969; Wagner *et al.*, 1974; Segal and Timiras, 1975; Dash and Patnaik, 1981), and a significantly higher mortality rate after exposure to cold in old animals when compared to young has been reported (Grad and Kral, 1957; Kiang-Ulrich and Horvath, 1979).

Although the reports described above show an alteration of the thermoregulatory response with aging, longitudinal studies of thermoregulation during late ontogeny are scarce, and the changes of this function with aging have not been fully explored. The purpose of the present experiment was to investigate changes in thermoregulatory function in male albino rats in a longitudinal study from young adulthood to old age.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Charles River, Inc., Boston, MA) were obtained at age 1½ months, weighing 160–170 g. Animals were housed individually in stainless steel cages in a room with temperature (24–25°) and humidity (50%) control and an alternate 12 hours light/dark cycle. All rats were given a chow diet and tap water *ad libitum*. Two weeks after their arrival, animals were taken to the experimental room and allowed to adjust to the laboratory conditions and experimental manipulation. Studies were performed at ages 3, 6, 9, 13 and 24 months. At these times, body weight, tail and body (from occiput to tail) length were measured. Body temperature (core and shell) at ambient temperature (24–25°) and oxygen consumption (VO_2) in the Thermal Neutral Zone (TNZ) (29 ± 1.0°) were measured. From 12 to 24 rats were used for every age point.

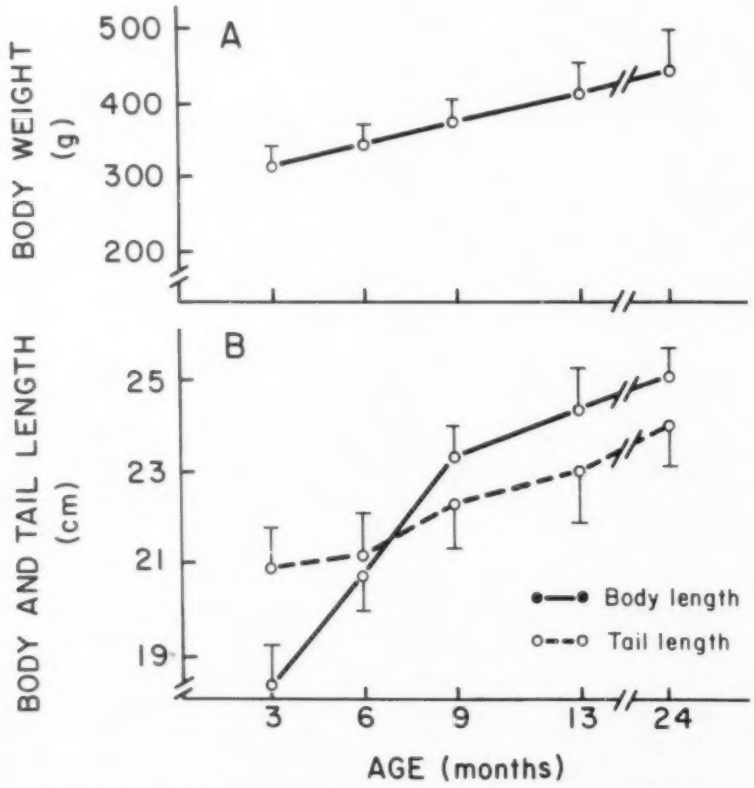

FIG. 1. Changes in body weight (A) and body and tail length (B) in rats from 3 to 24 months. Bars represent SEM. Numbers of animals in each group were 24, 24, 18, 18 and 12 for 3, 6, 9, 13 and 24 month groups, respectively.

Mild cold challenge

After obtaining the baseline data, animals were placed in a chamber at 18–19° and acclimated for 30 minutes. During the next hour, VO_2 was measured. The experimental procedure has been previously described (Balmagiya, 1975; Balmagiya *et al.*, 1975a).

Nonshivering thermogenesis

Nonshivering thermogenesis was determined by norepinephrine injection in the TNZ. These studies were performed two to three days after cold challenge to give the animals the opportunity to return to a normal basal metabolic rate. Measurements of VO_2 were made on sodium pentobarbital-sedated rats (30 mg/kg i.p.) in TNZ before and after i.m. norepinephrine injection (Levophed bitartrate sodium chloride solution Breon Laboratories, Inc., New York, N.Y.) in doses of 0.4 mg/kg body weight.

Thermal conductance and circulation index

Thermal conductance (C) expressed in units of weight-specific oxygen uptake (Aschoff, 1981) and thermal circulation index (i), indicating the "vessels component" of thermoregulation (Richards, 1973) were calculated for each animal from the experimental data by the following formulas:

$$C = \frac{M}{T_{rect} - T_{air}},$$

where M is a heat flow expressed as ml O_2/g/hr, T_{rect} = rectal temperature, T_{air} = air temperature.

$$i = \frac{T_{tail} - T_{air}}{T_{rect} - T_{tail}},$$

where T_{tail} is tail temperature, T_{rect} is rectal temperature and T_{air} is air temperature.

Statistical analysis

The differences between groups were tested using Student's t test for unpaired data. Differences were considered significant when p was less than 0.05.

RESULTS

Growth and resting metabolic rate changes

Body weight and tail and body length increased with aging. Body length increased during the first 9 months at a rate higher than body weight and tail length and then the increase slowed down (Fig. 1). VO_2 measured in the TNZ showed a significant decline between 3 and 6 months of age. VO_2 remained stable from 6 to 9 months of age after which it kept declining at a slow rate (Fig. 2).

Body temperature

Reports of core temperature changes during aging are confusing. The majority of authors suggest that it is unchanged (Shock, 1977; Altland and Rattner, 1981). Other studies have found fluctuating changes throughout life (Barrows and Kokkonen, 1978). Habicht (1981) reported an increase in core temperature from a young age to senescence with a later significant decline in old age.

In this study, changes in rectal temperature generally followed changes in resting metabolism in the TNZ. Rectal temperature decreased from 3 to 6 months, remained stable from 6 months to 9 months, and then dropped significantly from 9 to 13 months. On the average the decline in rectal temperature between 3 and 24 months of age was 0.8°C (Fig. 3).

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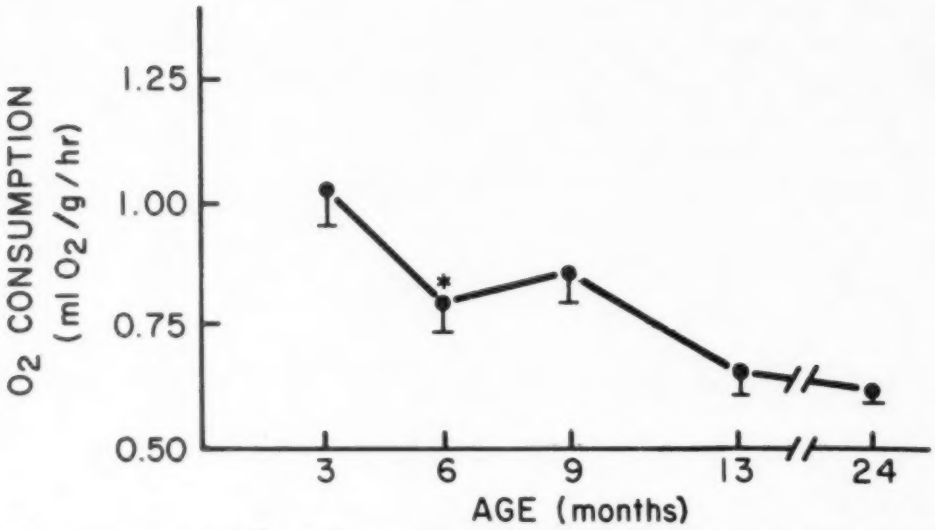

FIG 2. Oxygen consumption ($\bar{X} \pm$ SEM) in rats from 3 to 24 months. *Significantly different from previous age group ($p < 0.005$). The number of animals in each point is as described in Fig. 1.

Thermal conductance (C) and thermal circulation index (i)

Thermal conductance is a measurement of the ease of heat transfer from the body by radiation, conduction, convection, and evaporation and it is the reciprocal of insulation (Bradley and Deavers, 1980). It has been shown that thermal conductance in homeothermic organisms is negatively correlated with body weight (Scholender *et al.*, 1950; MacNab, 1980; Bradley and Deavers, 1980; Aschoff, 1981). In the present study, conductance decreased from 3 to 6 months, then remained almost unchanged from 6 to 13 months, and finally decreased slightly by 24 months of age (Fig. 4).

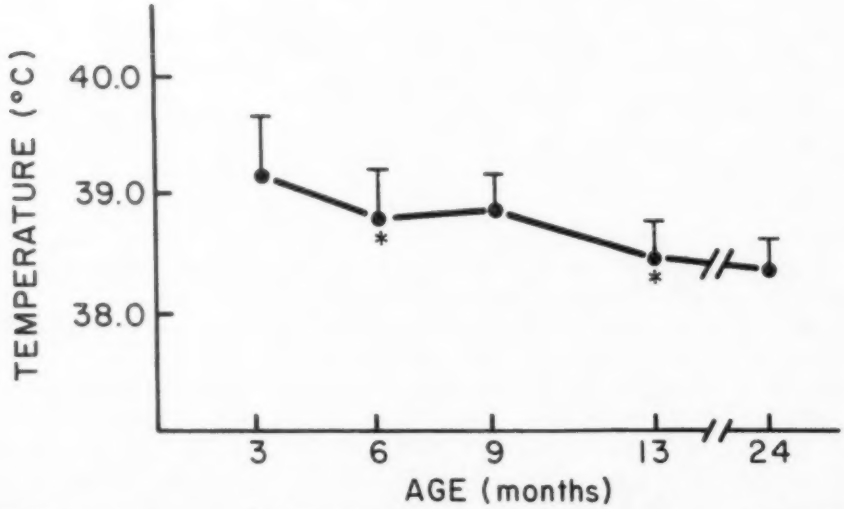

FIG. 3. Rectal temperature ($\bar{X} \pm$ SEM) in rats from 3 to 24 months. *Significantly different from previous age group ($p < 0.001$). Number of animals in each point is as described in Fig. 1.

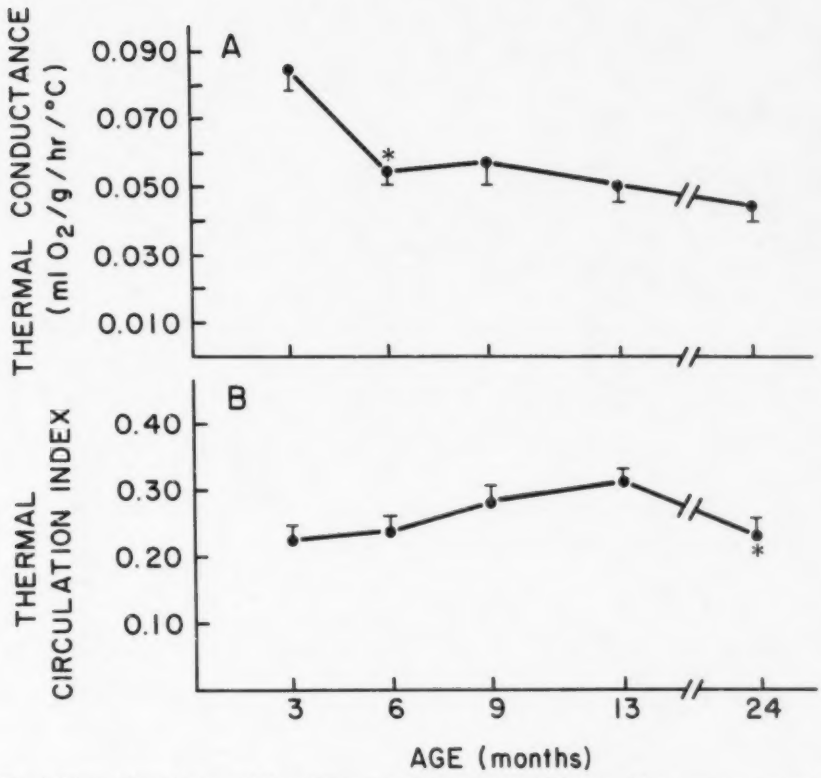

FIG. 4. Thermal conductance (A) and Thermal circulation index (B) for ambient temperature range 24–25°C in rats ($\bar{X}$ ± SEM) from 3 to 24 months. *Significantly different from previous age group ($p < 0.001$). Number of animals in each point is as described in Fig. 1.

Thermal circulation index measured at room temperature (24–25°C) rose slightly from 3 to 13 months, and dropped thereafter from 13 to 24 months to a level similar to 3 month old rats (Fig. 4).

Response to mild cold challenge

The increase in oxygen consumption during a mild cold challenge was proportional to resting oxygen consumption up to 13 months of age. At 24 months of age, the increase in oxygen consumption after a mild cold challenge was significantly higher than at earlier ages (Fig. 5).

The decrease in rectal temperature in rats during a cold challenge was the same from 3 to 13 months of age. At 24 months of age this decrease was more pronounced although not significantly different than at earlier ages. The decrease in tail temperature was similar from 3 to 9 months and became significantly larger at 13 and 24 months (Fig. 6). The recovery time following the cold challenge was directly correlated to age for both rectal and tail temperature (Fig. 7).

Nonshivering thermogenesis

The increase in VO_2 after norepinephrine injection is shown in Fig. 8. The ability to increase VO_2 after injection declined as age progressed. This decline was more marked

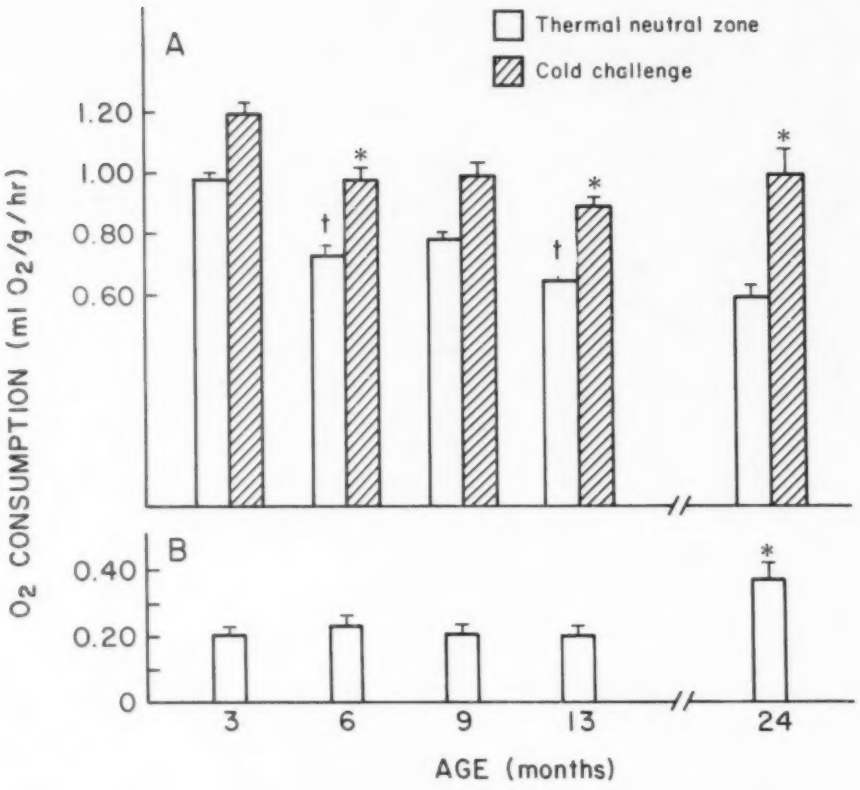

FIG. 5. Thermogenic response expressed in VO_2 to a mild cold challenge (18–19°C). Oxygen consumption in the TNZ and in cold in rats ($\bar{X} \pm$ SEM) from 3 to 24 months. On the bottom the differences in VO_2 between TNZ and cold expressing extra heat production during cold challenge. †Significantly different from previous age group ($p < 0.001$) in TNZ. *Significantly different from previous age group ($p < 0.001$) for cold. Number of animals in each group is 24, 24, 12, 9 and 9 for 3, 6, 9, 13 and 24 months, respectively.

between 3 and 6 months of age. It is interesting to note that heat production, after sympathetic system stimulation, exceeded heat production in rats during moderate cold challenge in all age groups, except in 24 month-old rats, where the relationship was reversed.

DISCUSSION

Present data indicate that resting metabolism in rats in the TNZ decreased about 47.5% between 3 and 24 months of age, which is in agreement with other reports (Denkla 1974; Sacher and Duffy, 1979). In our study we found a stable period from 6 to 9 months, during which VO_2 was practically unchanged. At the same time there was a definite and gradual decrease in rectal temperature during aging.

As described previously, age-related changes reported in the literature for rectal temperature are contradictory. In this study we found that rectal temperature changed in

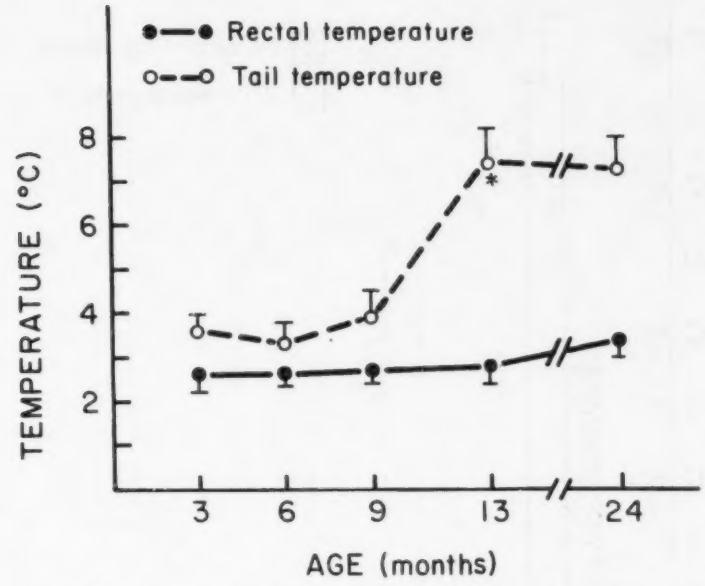

FIG. 6. Decrease in rectal and tail temperature after cold challenge ($\bar{X} \pm$ SEM) in rats from 3 to 24 months. *Significantly different from previous age group ($p < 0.001$). Number of animals in each group is as described in Fig. 5.

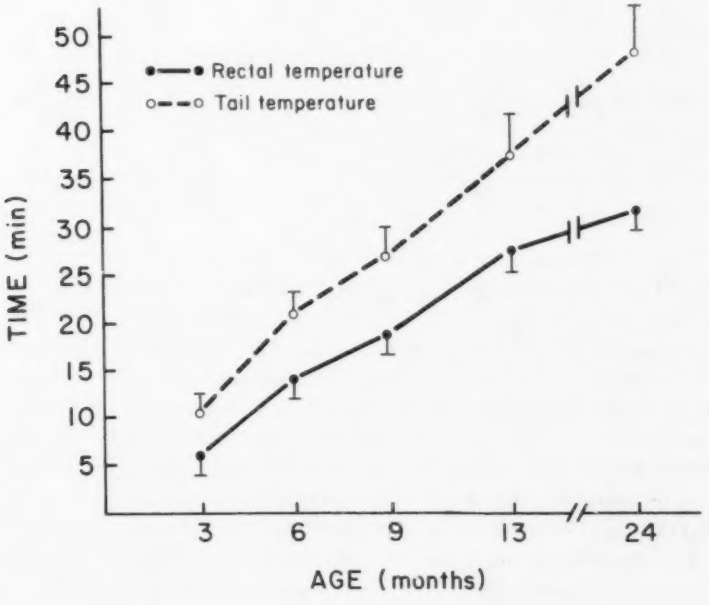

FIG. 7. Recovery time for rectal and tail temperature ($\bar{X} \pm$ SEM) for rats from 3 to 24 months. Every mean for recovery time is significantly different from previous age group ($p < 0.05$). Number of animals in each group is as described in Fig. 5.

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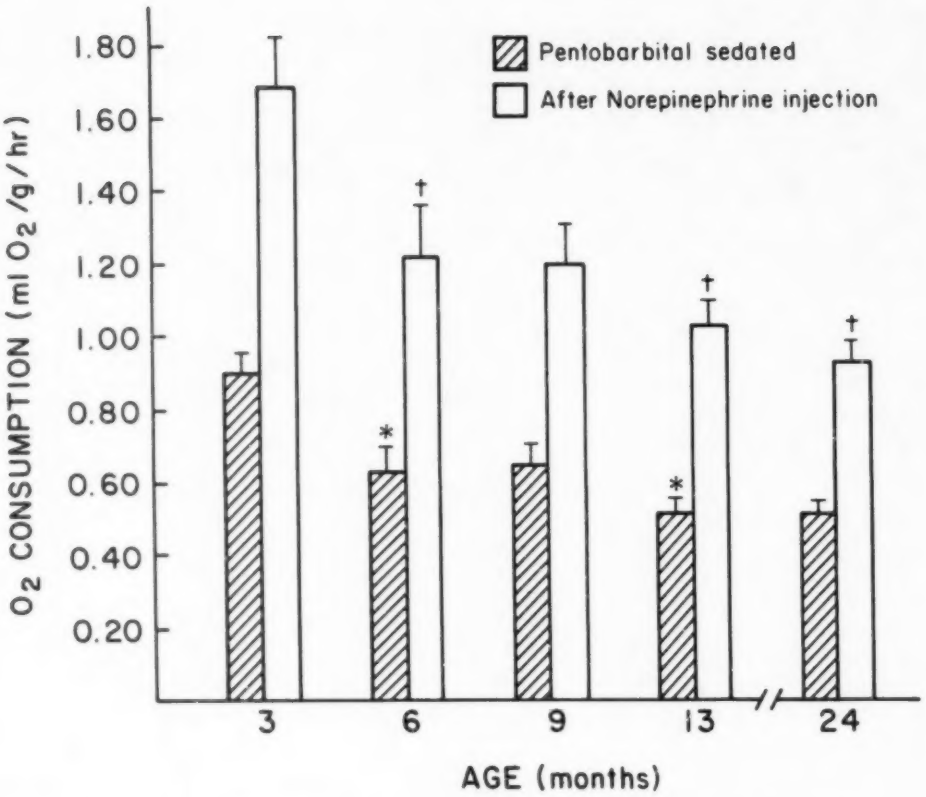

FIG. 8. Thermogenic response to norepinephrine stimulation expressed in VO_2 in TNZ ($\bar{X} \pm$ SEM) in pentobarbitol sedated rats from 3 to 24 months. *Significantly different from previous age group for VO_2 before injection ($p < 0.001$). †Significantly different from previous age group for VO_2 after NE injection ($p < 0.002$). Number of animals in each group were 24, 16, 16, 9 and 9 for 3, 6, 9, 13 and 24 months group, respectively.

parallel with VO_2. Thermal conductance in animals also decreased during aging, following almost exactly VO_2 changes, except for a decrease from 13 to 24 months, when thermal conductance dropped markedly. The age-dependent decline in thermal conductance could be an adaptive reaction aimed at reducing the rate of heat loss in the face of a declining heat production mechanism in order to maintain a constant body temperature. This has been proposed by Sacher and Duffy (1979) in their studies with mice. In contrast with our study, these authors did not find any decrease in rectal temperature as the animals aged. This discrepancy may be due to differences in experimental conditions since these authors measured rectal temperature only at an ambient temperature of 29° (the range of thermal neutral zone). Habicht (1981) who measured rectal temperature at an environmental temperature of 24°C (similar to ours) did find a statistically significant decrease in rectal temperature of 24 month-old mice in comparison to younger ones. Thermal conductance changes with aging were similar to changes in VO_2. In contrast, the thermal circulatory index (i) did not change significantly during aging. As was shown by Burton and Edholm (1955), the extremities and tail in animals are the principal sites of physical thermo-

regulation in an ambient temperature significantly below the thermal neutral zone. Since our measurements were performed at 24–25°C, the usual ambient temperature for these animals, and relatively close to their thermal neutral zone (TNZ), significant vessel constriction could not be expected. The decrease in the value of (i) from 13 to 24 months could be due to a general impairment in circulation, which has been previously described in aged humans and animals (reviewed by Wollner and Spalding, 1978). During moderate cold challenge physical mechanisms of thermoregulation are involved, vascular constriction becoming more important for two reasons: 1) the decrease in basal heat production, and 2) decreasing ability to produce extra heat by means of shivering (Collins *et al.*, 1977) and nonshivering thermogenesis (Jansky, 1979). In spite of the increase in vessel constriction with aging, the ability of rats of age 13 months and older to preserve their core temperature in the mild cold was diminished, their rectal temperatures during exposure falling more than twofold in comparison to young rats (Fig. 9). The time needed to return body temperature to normal after a mild cold challenge showed a constant increase during aging indicating a declining ability to maintain thermal homeostasis. Actually, it suggests that a temperature of 18–19° is not as "moderate" in the old animal as it is in the young.

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A still unanswered question is whether adult and especially old rats can produce heat by nonshivering thermogenesis. Brown adipose tissue (BAT) is important in nonshivering thermogenesis in the newborn and young adult (Dawkins and Hull, 1964; Bruk, 1979; Himms-Hagen, 1980; Davis, 1980). Some reports have claimed that adult humans (Hassi, 1977) and animals (Jansky, 1979) have no typical BAT and that shivering is their only cause of increased metabolism in response to cold (Wollner and Spalding, 1978). However, a recent report described the presence of BAT in middle age humans (Huttunen *et al.*, 1981). In the elderly it has been suggested that BAT is fully depleted, which can be a cause for hypothermia (Heaton, 1973).

A procedure to evaluate nonshivering thermogenesis is the measurement of heat pro-

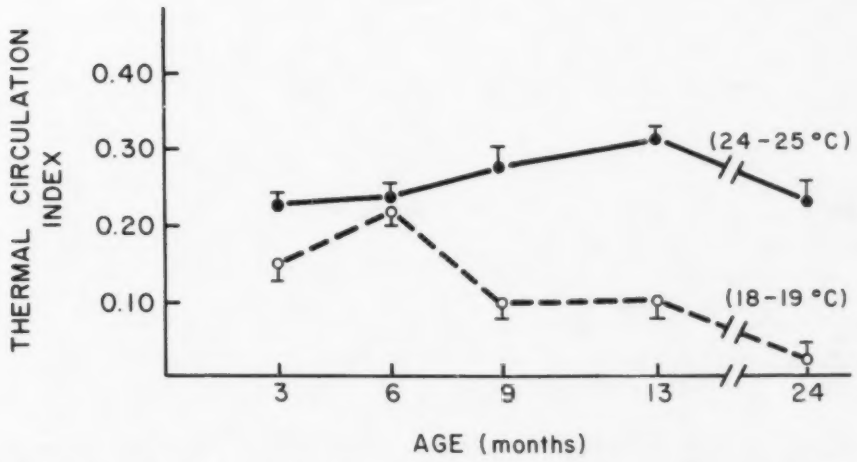

FIG. 9. Thermal circulation index for ambient temperature 24–25°C and 18–19°C in rats ($\bar{X} \pm$ SEM) from 3 to 24 months. The differences in means for warm and cold ambient temperatures are statistically significant ($p < 0.002$) for all age groups except 6 months old. Number of rats for each age point is as described in Fig. 1 (warm) and Fig. 5 (cold).

duction in the TNZ after norepinephrine injection (Jansky, 1979; Himms-Hagen, 1980). In our study, we used doses reported as giving a maximum effect in heat production in young rats (Jansky, 1979). Our results indicate that the ability to employ nonshivering thermogenesis during a mild cold challenge decreased with aging but it was still present at 24 months (Fig. 8). Nonshivering thermogenesis achieved by enhanced utilization of lipids has been found in middle-aged men during winter cold (Doi *et al.*, 1979) and in adult guinea pigs during cold acclimation (Hirvonen *et al.*, 1973; Huttunen and Kinnula, 1979). A recent study by Jessen (1980) showed the presence of nonshivering thermogenesis in humans of different ages (24–64 years) without any previous cold acclimation.

Additional heat production after NE injection might be produced in muscles (Ivanov, 1979), and/or in other organs and tissues (Depocas, 1960; Jansky, 1979) including white adipose tissue. Nonshivering thermogenesis has been described as "the first line of defense" against cooling acting before shivering occurs in zones close to thermoneutrality (Richards, 1973). Cold acclimation has been shown to increase the capacity for nonshivering thermogenesis (Balmagiya and Rabinovich, 1972; Richards, 1973; Bruk, 1979; Jansky, 1979). In our present study there is evidence that the amount of heat produced by rats in TNZ after NE injection exceeds the heat production during mild cold exposure in all age groups, except in 24 month-old animals. These studies suggest that the proportional contribution of shivering thermogenesis to heat production when exposed to a mild cold challenge is larger in old animals than in younger ones. However, the relationship between shivering and nonshivering thermogenesis in this situation still remains to be determined by direct measurement of shivering.

Accidental hypothermia in the elderly has been well established (Green, 1981). This study shows that the old organism has a reduced ability to produce extra heat after norepinephrine stimulation when compared to younger ones. In addition, when exposed to a mild cold challenge they seem to need more heat (evidenced by a larger increase in VO_2) to maintain body temperature. Nevertheless the decrease in core temperature in this situation is greater in older animals and it takes them a longer period of time to restore temperature to normal. Although the intrinsic mechanisms responsible for these altered responses is not known, they may contribute to the problem of overcooling in the elderly when exposed to temperatures just a few degrees below the thermoneutral zone.

SUMMARY

Body temperature and thermoregulation were studied in male Sprague-Dawley rats during aging. Both oxygen consumption and rectal temperature decreased from 3 to 24 months of age, with a stable period between 6 and 9 months. The ability to respond to a mild cold challenge (18–19°) was impaired in old rats when compared to young, especially at 24 months. These studies suggest that shivering thermogenesis may be the main source of heat production in the old organism when faced to a mild cold challenge.

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AGING CHANGES IN THE KIDNEYS OF TWO POECILIID FISHES, THE GUPPY *POECILIA RETICULATUS* AND THE AMAZON MOLLY *P. FORMOSA**

A.D. WOODHEAD,† V. POND and K. DAILEY

Biology Department, Brookhaven National Laboratory, Upton, NY 11973

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Abstract—Histological surveys of the kidneys of two poeciliid fishes, the Amazon molly *Poecilia formosa* and the guppy *P. reticulatus*, throughout their lifespans showed no regular aging trends. Few kidney lesions were found in the Amazon molly until very late in life, 60 months or more, when obsolescent glomeruli and dilated renal tubules occurred. Guppies showed involutional changes of the renal system earlier, and the lesions became more severe with age, particularly in male fish. Hemopoietic tissue was reduced in amount in older fish of both species. Guppies of a year and older had marked accumulation of melanin in the melanomacrophage centers of the kidney, and the amount present increased with age. By contrast, there was little melanin deposition in mollies until almost the end of the lifespan. Hyaline droplets were consistently seen in the renal tubules of the mollies, but were rare in guppies. The degenerative changes in the kidneys of these two teleosts are similar to those seen in the kidneys of aging mammals. Despite the loss of normal structure in older fish, it seemed unlikely that degeneration of the kidney was directly involved in aging and death.

INTRODUCTION

BIDDER HAS SUGGESTED that fish and other cold-blooded vertebrates which continue to grow after sexual maturity do not age and are potentially immortal (Bidder, 1932). Although this hypothesis was clearly disproven by Comfort (1960), the idea is still prevalent in the exact as well as in the epigrammatic literature (Kohn, 1971; Anonymous, 1982). Fishes as a class have no monopoly on longevity. Although there is a high degree of variability in the length of lifespan throughout the vertebrates, some mammals, birds, reptiles and amphibia, as well as fishes, live over 60 years (Comfort, 1979). Part of the misconception that aging in fishes and other poikilotherms differs from that in warm-blooded vertebrates may be due to the lack of information on cellular and tissue changes with age. The demonstration that there are anatomical alterations with age similar to those in the organs of mammals would give strong, if not conclusive, support to the concept of a common aging process in vertebrates.

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†To whom correspondence should be addressed.

We have made histological surveys of the changes in several organs throughout the life of guppies, Amazon mollies and Siamese fighting fish which have provided evidence of degeneration and dysfunction towards the end of their lifespan (Woodhead, 1979). The present paper describes the changes with age in the kidneys of the Amazon molly and the guppy. The work was based upon a collection of serial histological sections from guppies, generously donated to us by Dr. Alex Comfort, and from Amazon mollies taken from our laboratory population.

MATERIALS AND METHODS

The guppies were members of a population which had been maintained by Dr. Comfort under normal aquarium conditions into extreme old age; the fish had not been subjected to any radical changes in keeping conditions during their lives and were designated "normal growers" (Comfort, 1960). Our survey was based upon serial sections of 22 females and 20 males, ranging in age from 5 to 61 months.

We have been rearing populations of the Amazon molly in the laboratory for five years. The Amazon molly is a small, live-bearing and all-female poeciliid species native to southern Texas and Mexico. Females reproduce gynogenetically, that is, the eggs are activated and develop after mating with males of a closely related species. There is no genetic contribution from the male to the young; sperm provides only the stimulus initiating egg development. Our sample comprised 22 females, ranging in age from 1 to 59 months, and 5 postreproductive females over 60 months of age.

Serial sections were cut at 8 μm and routinely stained with Heidenhain's hemotoxylin and eosin; sections from 3 of the female guppies and 2 of the male guppies had been stained with Mallory's trichrome stain. An overall qualitative description of the kidney was given for each fish, and the occurrence of lesions, infections or tumors was noted. Measurements were made of the proportion of excretory, hemopoietic and reticuloendothelial tissue in the kidney of Amazon mollies of different ages by projecting an image of the section onto paper of standard thickness and tracing the outlines of the various tissues. These outlines were then cut out and weighed to determine the extent of the areas covered by the different tissues. The size of the kidney in relation to body size, and the size of the glomeruli, the width of the different segments of the excretory nephron and the heights of the epithelial cells lining the segments were measured directly through the microscope. As in previous studies, we adopted the age of two years as a time in the life cycle at which we could make an arbitrary but convenient distinction between "young" and "old" individuals (Woodhead and Ellett, 1966; 1967).

RESULTS

The structure and function of the fish kidney have been well documented and a major review by Hickman and Trump (1959) assembled much comparative information. This wealth of data has resulted from the excellent homology between the piscine and mammalian kidney, so that fish kidney and nephron preparations have been widely used in comparative studies of renal secretory transport (Trump and Bulger, 1968).

Our observations show that the kidneys of the guppy and the molly are paired, elongate and reddish-brown organs, lying beneath the vertebral column and extending the length of the body. At the anterior end (the head kidney) the organ is wide but narrows sharply at the level where the esophagus ends (the trunk kidney) and narrows again at the mid-body level, from where it is represented only by a slender thread of tissue. The kidneys are separate along most of their length, but a small bridge of renal tissue joins them towards the end of the body. In many species of fish, the head and trunk kidneys are anatomically and functionally separated, with hemopoietic (blood-forming) tissue predominating in the head kidney, while the trunk kidney contains a preponderance of excretory tissue. In the guppy and the molly, both components are represented throughout the kidney, though there is slightly more hemopoietic tissue in the anterior kidney.

Four major tissue elements are present in the fish kidney: the excretory nephrons, the hemopoietic tissue, the reticuloendothelial tissue, and the interstitial tissue. In addition, the kidney also contains endocrine tissues: the interrenal gland, the chromaffin tissue, and

the corpuscles of Stannius; aging changes in the interrenal and chromaffin tissue of the guppy have already been described (Woodhead and Ellett, 1967). The excretory nephron consists, in succession, of a renal corpuscle, composed of a glomerulus with a wide lumen and thin capillary walls together with the enclosing Bowman's capsule leading into a short, highly ciliated neck region. There is a sharp demarcation between the neck segment and the first proximal segment which follows, recognizable by the columnar epithelium with coarsely granular cytoplasm and a prominent brush border. The second proximal segment, with cuboidal epithelial cells with numerous mitochondria, but a less well-defined brush border follows, then there is a very short, ill-defined, ciliated intermediate section, and finally a distal segment with clear, low columnar epithelial cells and eosinophilic cytoplasm. This system of tubules leads into the collecting duct system.

A typical representative section through the kidney of a young molly is shown in Fig. 1; this section is also illustrative of young guppies. The glomeruli are large, with flower-like tufts of capillaries, filling Bowman's capsule. Bowman's space is narrow. The neck seg-

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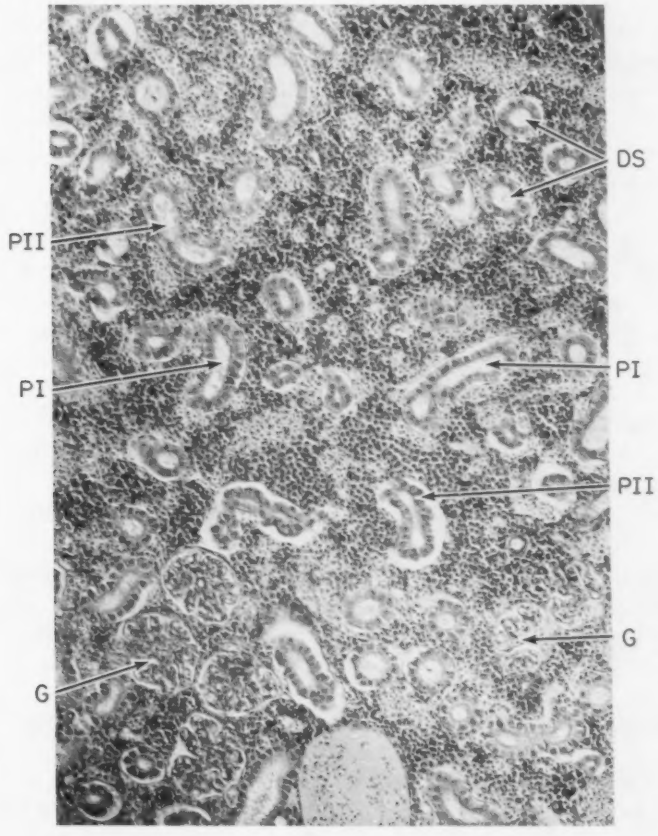

FIG. 1. A section through the kidney of a young Amazon molly aged 4 months showing glomeruli (G), first proximal segments (PI), second proximal segments (PII) and distal segments (DS). There is abundant hemopoietic tissue between the nephrons. × 200. H & E.

ment, proximal and distal tubules are lined with regular cuboidal epithelial cells, and there is abundant hemopoietic tissue between the nephrons. Very occasionally in young fish there was an empty space between the nephron (particularly the distal segment) and hemopoietic tissue; this feature was probably a result of shrinkage during fixation.

The kidney is an important hemopoietic organ in fishes, and the nephrons are surrounded by interstitial tissue, which supports the blood-forming elements. Hemoblasts, immature blood cells and mature red blood cells can be distinguished. The free and fixed macrophages of the reticuloendothelial system surround sinuses through which blood from the renal portal vein passes; they remove particulate matter, metabolic wastes and old blood cells from the circulation (Ellis *et al.*, 1978; Roberts, 1978). Yellow-brown macrophage centers also lie within the interstitial tissue and in both species, the centers are bounded by a thin connective tissue capsule.

There were several consistent differences between the kidneys of guppies and mollies, which were evident even in young fishes, and became more pronounced in older individuals. There was much greater deposition of melanin in the melanomacrophage centers of the kidneys of guppies, a difference which was also reflected in the melanomacrophage centers of the spleen and in the phagocytes of the atrium of the heart. In young guppies under 8 months, discrete melanin granules occurred in the golden melanomacrophage centers, but by one year, large droplets were present, and in many older guppies over 2 years, the centers were laden with intense black pigment. By contrast, there was little deposition of melanin in the melanomacrophage centers of the young mollies, and melanin became conspicuous only in the very oldest fish in our series, aged 60 months and more. Deposition of melanin in the melanomacrophage centers of these postreproductive fish coincided with the appearance of melanin spots on the tail (Woodhead and Setlow, 1980). It has been suggested that the extent of melanin deposition in different fish species may be related to inherent differences in lipid metabolism or to differences in the diet (Roberts, 1978). A second distinguishing feature between the 2 species was the presence of brightly staining hyaline droplets in the epithelial cells of the proximal tubule of many of the mollies surveyed, both young and old. These droplets were far less frequently seen in guppies. In mammals, hyaline droplets are an indication of lesions in the glomerulus, but this is thought to be unlikely in fishes (Roberts, 1978). The glomeruli of affected mollies appeared normal.

Uroliths occurred in the excretory tubules of a 5-month-old male guppy and were encountered in the majority of guppies aged 1 year and older. Very few uroliths were seen in Amazon mollies. The material forming the urolith, thought to be calcium or magnesium salts within an organic stroma, stained deep purple with hematoxylin. The formation of uroliths may be related to a high level of carbon dioxide in the water, to dried food or to an imbalance of calcium and magnesium in the water, and are often seen in fish kept in intensive culture.

The kidneys of several young and old individuals of both species were infected with *Mycobacterium,* usually occurring in the hemopoietic tissues; both soft and hard focal granulomas were present. Generally, the extent of infection increased with age. In 2 old guppies, there was some fibrosis of the hemopoietic tissue around the tubercles. These lesions have been described in detail, since fish tuberculosis is a common problem in species kept in aquaria (Wolke, 1975).

There was little anatomical evidence of any marked degeneration in the kidneys of young mollies, but small lesions occurred in the kidneys of young guppies of both sexes.

Generally, degeneration of the kidney was more pronounced in male guppies, and its onset was earlier, in fact, only the 4 youngest males (under 8 months) in our series of 20 males had kidneys which we classified as normal. Female guppies showed onset of degenerative features from about 24 months. The lesions seen in the young and old guppies were anatomically similar but were more extensive in older fishes.

Aging of the kidney of the Amazon molly

There were only limited changes with age in the kidneys of Amazon mollies until towards the end of their lifespan; 2 of 5 senile fish over 60 months showed some degenerative features, although the other individuals showed rather few changes. There was no reduction in the size of the kidney nor in the extent of excretory elements (Table 1) in older fish; measurements of the different segments of the nephron also showed no differences between young and old, except for the neck segment, which tended to be longer and to be lined with larger cells in old individuals (Table 2). Dilation of the first and second proximal segments was a fairly common feature of the kidney of older mollies, but the dilation was usually confined to one or two nephrons at the anterior of the kidney, and did not occur generally throughout the organ. There was very little deterioration of the glomeruli. Hemopoietic tissue appeared to be quite normal, except in 1 molly of 49 months which showed widespread areas of fibrosis and necrosis replacing the blood-forming tissue. A comparison of the extent of hemopoietic tissue in young and old mollies (including the 5 fish over 60 months) showed that there had been a reduction in old fish (Table 1); the difference was significant at the 1% level. There was not, however, a consistent trend in the loss of hemopoietic tissue with age.

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TABLE 1. A COMPARISON OF THE AREAS OF HEMOPOIETIC AND EXCRETORY TISSUE IN SECTIONS OF THE KIDNEY FROM YOUNG AND OLD AMAZON MOLLIES.

Amazon mollies	No. of Fish	Excretory tissue Mean ± S.E.	Hemopoietic tissue* Mean ± S.E.
Young	9	25.7 ± 1.9	63.6 ± 2.7
Old	9	29.4 ± 1.8	60.0 ± 1.8

*Young and old differ significantly at the 1% level ($p = .0088$).

TABLE 2. COMPARISON OF THE SIZE OF THE EPITHELIAL CELLS IN DIFFERENT SEGMENTS OF THE EXCRETORY NEPHRON IN YOUNG AND OLD MOLLIES.

Age of fish	Neck segment (length/height epithelial cells) μM	First proximal tubule (height epithelial cells) μM	Second proximal tubule (height epithelial cells) μM	Distal tubule (height epithelial cells) μM	Average number of tubules/ microscopic field μM
Young fish 1–24 mo.	51/5	19	17	16	6
Old fish 25–60 mo.	88/17	20	18	18	5

There were some conspicuous lesions in the kidneys of 2 of the 5 senile mollies over 60 months of age (Fig. 2*a,b*). In particular, there was marked glomerular obsolescence. The degree of atrophy of the glomeruli varied considerably in the same kidney; some glomeruli

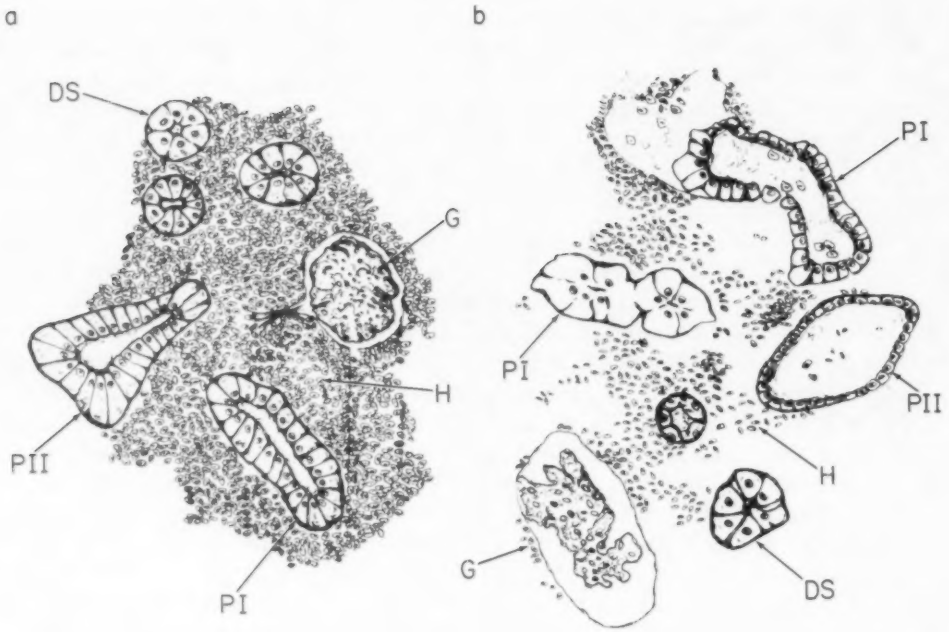

FIG. 2. Drawing of a section through the kidney of (*a*) a young and (*b*) a senile Amazon molly, showing glomeruli (G), first proximal segment (PI), second proximal segment (PII) and distal segment (DS). Occasionally in old mollies the hemopoietic tissue (H) is sparse, the glomeruli shrunken and the tubules distorted.

exhibited only a wrinkling of single capillaries, accompanied by slight thickening of the visceral basement membrane, while at the other extreme, the entire capillary tuft had been reduced to a small shrunken mass of tissue, with few spaces (which may have been vascular channels) traversing the shrivelled remains. Hyaline material occupied the resulting space in Bowman's capsule.

Dilated tubules were also present in the head kidney of the these two old fish and involved all segments of the nephron. In parts of both proximal and distal segments the epithelial cells had swollen, burst and desquamated, shedding cell debris into the lumen of the tubules. Hemopoietic tissue was somewhat sparse, leaving intercellular spaces around the tubules which were frequently filled with a clear fluid. There was enhanced deposition of melanin within the melanomacrophage centers.

Aging changes in the kidneys of the guppy

The lesions which had their onset in male fish from 8 months of age and in females of 2 years tended to become more extensive and extreme with the passage of time. There was no well-defined trend of changes in any particular feature with age, although the extent of all the lesions was greater in older guppies and was particularly marked in fish over 60 months. Obsolescent glomeruli, dilated tubules, uroliths, intense deposition of melanin and loss of hemopoietic tissue occurred singly or collectively. The most prevalent lesion observed was dilation of the nephron (cystic disease), ranging from swelling of the proximal segments of the few tubules, to a massive dilation of most segments of many tubules,

so that the interstitial and hemopoietic elements were represented only by a thin outer rim of tissue (Fig. 3a,b). Many intermediate stages of tubular dilation and degeneration were seen, from the early stages in which a few epithelial cells had been shed into the lumen of the nephron, to later stages in which most of the lining cells had been sloughed off and numerous cell casts were seen, lying in a brightly staining pink exudate. There was no compensatory lengthening of unaffected parts of the nephron nor hypertrophy of existing cells, as occurs in mammals (Barrows *et al.*, 1960).

In contrast to the Amazon mollies in which degeneration of the glomeruli occurred only in the senile postreproductive individuals, shrunken glomeruli were found in all old guppies. The number affected varied from fish to fish, and again there was no clear-cut increase with age, although in the guppies over 60 months the majority of glomeruli were damaged. The glomerular lesions ranged from wrinkling of the capillary membranes to complete shrinkage and hyalization of the glomerular tuft (Fig. 4).

There was little change in the condition of the hemopoietic tissue in old guppies, except for those fish over 60 months, where it had become very sparse. The amount was also

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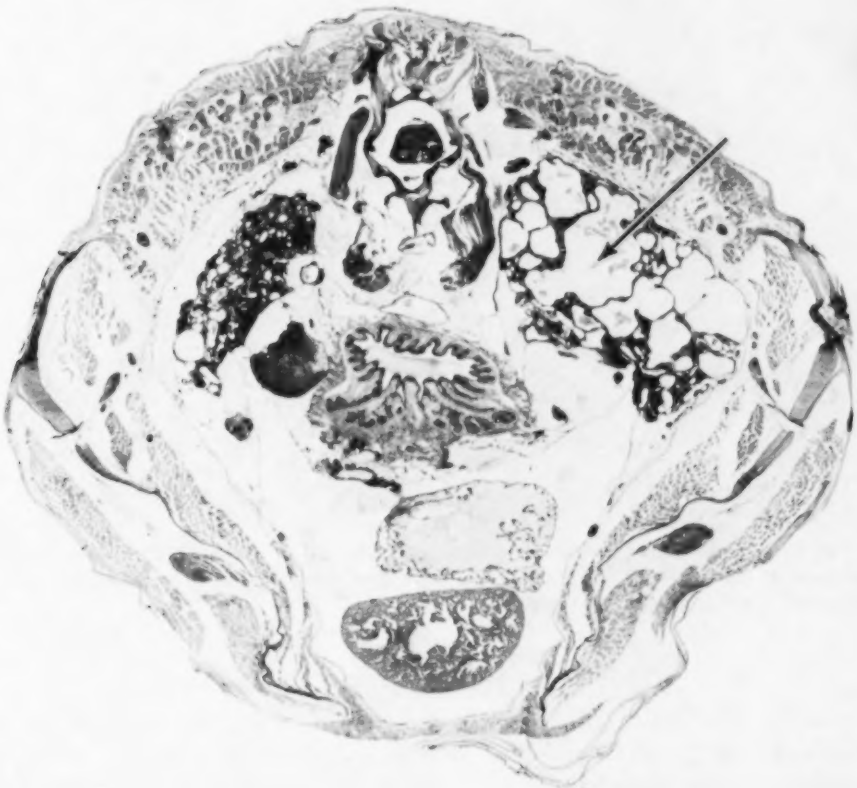

FIG. 3a. Transverse section through the body of a male guppy, aged 49 months, showing massive dilation of the tubules of the kidney (arrow). × 60. Masson's Trichrome.

reduced in fish showing extreme tubular dilation, but even in those areas where there was widespread tubular dilation and necrosis, the adjacent hemopoietic tissue appeared normal. There were no renal tumors found in either species.

FIG. 3b. Higher magnification of a section of the kidney of a 52-month-old male guppy showing massive tubular dilation (arrow). The fish had a large, fully ripe testis, parts of which are seen at the lower edge of the photomicrograph. × 600. Masson's Trichrome.

DISCUSSION

Glomerular and tubular lesions similar to those which have been described for mammals (Shock, 1956) were seen in the kidneys of old Amazon mollies and guppies. Although there were no regular aging trends in any one individual feature of the kidney, the extent of the lesions was greater in older fish. Changes in renal morphology in the molly did not become marked until the end of the reproductive lifespan. Guppies, however, showed involutional changes at a much earlier age, and the degeneration of the

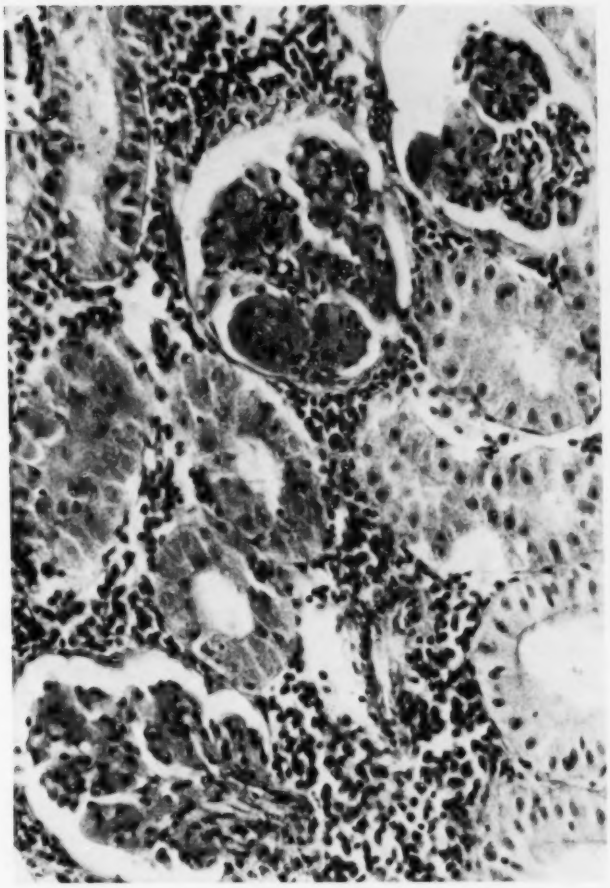

FIG. 4. Photomicrograph of section through the kidney of a 50-month-old female guppy showing 3 glomeruli in different stages of obsolescence. × 600. H & E.

kidney was more severe. Whether the differences between the two species are inherent or associated with environmental conditions remains undetermined.

There is a well-marked decline in renal mass and function in aging humans. The changes may involve a number of anatomical sites such as the glomeruli, tubules, interstitium and blood supply. So far, it has proved difficult to resolve the degree to which the changes with age result from involution of the kidney itself, or are indirect effects due to more widespread aging changes in the human body in general, particularly in the vascular system. Many investigators maintain that vascular changes in the blood supply to the nephrons in humans are the primary cause of kidney degeneration and that the distribution of vascular lesions in turn determine the site and extent of other aging effects in the kidneys. In either case, it is agreed that a decline in renal function does not play a major role in the phenomonology of old age (Shock, 1956). Human kidneys have a great

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reserve capacity and while they are equipped to receive 20 to 25% of the output of the heart, they can function adequately on 5 to 10%. Despite marked vascular changes, including narrowing of the capillaries, persistent constriction of vessels, and loss of vessels, internal homeostasis is maintained under basal resting conditions (Cohn and Shock, 1949; Elkington and Danowski, 1955; Shock, 1956; Korenchevsky, 1961). We have no data as yet on the extent to which vascular changes in old age are responsible for nephron dysfunction and degeneration in the molly and the guppy, although in some postreproductive fish there were degenerative changes in the heart muscle, and a marked loss of muscle fibers in the bulbous arteriosus.

The fish kidney is concerned with the excretion of divalent ions and with the regulation of salt and water homeostasis; toxic nitrogenous end products are eliminated via the gills and intestine. Extensive tubular and glomerular lesions may develop before the fish becomes morbid. Guppies with large dilated tubules and many obsolescent glomeruli supported large ripe gonads (Table 3); generally, females with compromised kidneys carried the same number of ripe eggs as females of the same size with relatively normal kidneys. Similarly, the presence of large deposits of melanin in the melanomacrophage centers or of large granulomas throughout the kidney did not appear to be life-threatening.

There have been very few studies of structural changes in the kidney of fish throughout their lifespan. Markofsky and Milstoc (1979) described changes with age in the kidney of the male annual fish *Nothobranchius guentheri*. The changes included some tubular dilation, thickening of Bowman's capsule and accumulation of lipochrome, but, overall, the lesions were not marked. Pacific salmon, semelparous species, are particularly interesting as they show "parental death," which has been equated with accelerated aging. Postspawning salmon show kidney lesions including sclerosis of the glomeruli, thickening of Bowman's capsule and tubular degeneration (Robertson and Wexler, 1960, 1962).

In their discussion of the evolution of the fish kidney, Faith and Trump (1966) and Hickman and Trump (1959) have stressed the essential similarities between excretory nephron of fishes, amphibia and mammals. They drew attention to the homology between the pattern of degeneration of the glomeruli during ontogeny of aglomerular fish (the daddy sculpin) and glomerular degeneration in human kidney disease. Our findings sug-

TABLE 3. LACK OF AN EFFECT OF EXTENSIVE TUBULAR DEGENERATION ON THE ABILITY TO SUPPORT A LARGE GONAD IN MALE AND FEMALE GUPPIES.

Sex	Age (mo.)	Kidney State	Gonad size as a % of body size
Female	5½	Normal	31
		Degenerate	30
	9	Normal	30
		Degenerate	34
	28	Normal	21
		Degenerate	19
Male	8	Normal	19
		Degenerate	22
	15	Normal	24
		Degenerate	21
	37	Normal	26
		Degenerate	29

gest that the homology between fish and mammals may be extended to the pattern of changes in the excretory system with the advent of aging.

SUMMARY

This paper describes the changes in renal morphology in laboratory-bred populations of the Amazon molly and the guppy throughout life. There was little evidence of marked degeneration of the kidney of the Amazon molly until very late in life, 60 months and older, when dilation of the renal tubules and obsolescence of the glomeruli were noted. Guppies showed involutional changes much earlier, and the degeneration was more severe, especially in the male fish. Hemopoietic tissue was less extensive in old fish, but no definite trend of loss was seen with increasing age.

Guppies showed a marked accumulation of melanin in the melanomacrophage centers of the kidney, the degree of which increased throughout life. There was little melanin deposition in Amazon mollies until the end of the lifespan; this deposition coincided with the appearance of melanin spots on the tail. Another difference between the 2 species was the presence in the Amazon molly of hyaline droplets in the renal tubules which were rare in the guppy. The degenerative changes in glomeruli and tubules of the nephron of the Amazon molly and guppy were similar in appearance to those seen in the kidney of mammals. Despite loss of normal kidney structure in older fish, it seemed unlikely that degeneration of the kidney was directly involved in aging and death.

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AGEING OF HAMSTER EMBRYO FIBROBLASTS AS THE RESULT OF BOTH DIFFERENTIATION AND STOCHASTIC MECHANISMS

M. RAES and J. REMACLE

Laboratoire de Biochimie Cellulaire, Facultés Universitaires, 5000 Namur, Belgium

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Abstract—Fibroblasts from hamster embryos were serially cultivated *in vitro* and their evolution followed from a morphological, physiological and biochemical point of view. After an exponential growth for about 20 passages, cells entered the ageing phase which ended up after 29–34 passages. From our observations, it seems that the arrest of growth results from two different phenomena: first, the typically fibroblastic cells may undergo a stochastic ageing process; second, some of these cells evolve into a terminal differentiation process, characterized by a different non-fibroblastic phenotype.

INTRODUCTION

THE ORIGINS of cellular ageing are not yet understood even if many attempts have been made to tackle this basic problem of life; in particular the question as to whether ageing is under the control of a genetic program or is influenced by extrinsic factors is not yet resolved and is the subject of many hypotheses (Orgel, 1973; Martin *et al.*, 1974; Wright and Hayflick, 1975; Holliday *et al.*, 1977; Bell *et al.*, 1978; Grove and Cristofalo, 1978; Harley and Goldstein, 1978; Shmookler-Reiss *et al.*, 1980; Smith and Whitney, 1980). One approach to this problem is the comparative study of ageing using living material provided by nature. Such studies have been extensively carried out on animals and they have brought much valuable information. The interpretation of these data in view of the question raised is, however, difficult because the answer is always reported at a cellular or even at a molecular level (Lamb, 1977).

Cell culture is a well studied *in vitro* system of ageing because of its reproducibility, its well controlled experimental conditions and its relationship to *in vivo* ageing (Hayflick, 1974; Martin *et al.*, 1970; Schneider, 1978). However most of the cell culture studies have been carried out on human cells; the best characterized cell lines are WI-38 (Hayflick and Moorhead, 1961), MRC-5 (Jacobs *et al.*, 1970), IMR-90 (Nichols *et al.*, 1977) and TIG-1 (Ohashi *et al.*, 1980), all from human sources. When used in ageing research, cells from other animal species are studied in a specific context and are never fully characterized. Only cells from chicken embryos have been used in several reports (Hay and Strehler, 1967; Lima and Macieira-Coelho, 1972; Ryan, 1979).

We thought that an extensive, comparative study of cell lines from different animal sources but cultivated in the same conditions, could bring some information on the respective importance of the environment and the genetic program in cellular ageing. In this line, we tested fibroblasts from different species and found fibroblasts isolated from embryonic hamster lungs the most interesting. The hamster has a short life-span and its fibroblasts could attain around 31 population doubling levels (PDL) in culture. Moreover, their *in vitro* ageing is linked to the appearance of new phenotypic cells which allows us to distinguish between the differentiation and the ageing process. In this paper, we show that both processes can explain the arrest in the cell division and are superimposed during cell culture. For these reasons, embryo hamster fibroblasts in culture represent a new interesting tool for investigations on the mechanism of cellular ageing.

METHODS AND PROCEDURE

Cell culture

Medium. The growth medium used was Eagle's minimum essential medium with non-essential amino acids and Hanks' buffered salt solution, with twice the concentration of vitamins and amino acids (Eurobio, Paris, France); 3.6 g of $NaHCO_3$ and 250 mg of aureomycin (Lederle, S.A., Belgium) were added per 5 l and the final pH was 7.15. Before use, the medium was supplemented with 10% foetal calf serum (Bio-Lab Ltd., Israel).

Isolation of primary cells. Fibroblasts were isolated from the lungs of 13–16-days-old Syrian hamster (*Mesocricetus auratus*) embryos. The tissue was first minced with paired scalpels into 1 mm^3 pieces. These tissue fragments were enzymatically treated by different methods: with trypsin (Rubin, 1973), collagenase (Hilfer, 1973), and with a hyaluronidase-collagenase mixture (Prop and Wiepjes, 1973). As expected, considering the fibrous structure of the pulmonary tissue, the collagenase-hyaluronidase mixture gave better yields. The primary culture was cultivated into 75 cm^2 Falcon flasks (Falcon Plastics Ca, U.S.A.). Cells from the first confluent culture were considered, for convenience, at a population doubling level of 2 (PDL 2). Enzymes came from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Secondary cells

As soon as the primary culture was confluent, cells were trypsinized and subcultivated into 75 cm^2 Falcon flasks, following a constant subcultivation schedule with a 4:1 split ratio (Hayflick and Moorhead, 1961). When the rate of cell growth began to decrease, a 2:1 split ratio was used.

At different stages of the cell culture, cells were stored in liquid nitrogen in medium containing 10% dimethylsulfoxide (Shannon and Macy, 1973). The reconstituted frozen cells behaved like non-frozen cells despite a 10–50% cell loss after thawing. The population doubling level was corrected for this loss. Cultures were checked for mycoplasm contamination and the results were negative (Chen, 1977).

Cloning

In the cloning experiments, we used the RPMI-1640 medium (Eurobio, Paris, France) which is well adapted for hamster foetal lung fibroblasts (Emura *et al.*, 1979). The experimental procedure was as follows: single cells (from 100–200) were plated into 60 mm tissue culture Petri dishes (Falcon Plastics, CA, U.S.A.) with 4 ml of RPMI-1640, supplemented with 15% foetal calf serum. Every 2 days, half the medium was renewed with fresh medium supplemented with 20% foetal calf serum. After 10 days incubation in a 5% CO_2/95% atmosphere at 98–100% relative humidity, cells were fixed and stained with Giemsa (Merck, Darmstadt, Germany). The number of cells in each colony was determined (at least for colonies of less than 256 cells) and their morphology examined. Plating efficiency represents 100 times the ratio of colonies per cells seeded (Reid, 1979).

Thymidine incorporation

We determined the Cristofalo index (Cristofalo and Sharf, 1973), i.e., the percentage of cells incorporating [^{3}H] thymidine (NEN, England) during 24 h, at different stages of the cell culture. A minimum of 500–800 cells was counted for each determination.

Light microscopy

Cells were systematically examined *in situ* with a Leitz Diavert (Germany) phase contrast microscope at different days after subcultivation and at different stages of the cell culture.

Transmission electron microscopy

We tested several embedding procedures in order to obtain a good morphological preservation of cultivated cells embedded *in situ*; the following procedure gave the best observations.

Cells were first rinsed 3 times in a 0.05% $MgCl_2$, 0.1 M potassium phosphate buffer, pH 7.4 at room temperature. Concerned with the conservation of microtubules, we excluded the use of a cacodylate buffer. Indeed, sodium cacodylate, an arsenical derivative, is known to inactivate the mitotic spindle (Piton, 1929; Dustin, 1938). More recently, Luftig *et al.*, 1977 pointed out that the use of cacodylate in buffer solutions for electron microscopy may lead to an extensive dissassembly of microtubules. After being rinsed, cells were fixed in 2% glutaraldehyde (BDH Chemicals, England) in 0.05% $MgCl_2$, 50 mM potassium phosphate for 30 min. Fixation was carried out at room temperature for 10 min, and afterwards at 4°C. After a wash (3 × 5 min at 4°C) in a 0.1 M potassium buffer, pH 7.4, cells were post-fixed for 30 min (4°C) in 1% osmium tetroxide (LADD Research Ind., U.S.A.) dissolved in the same buffer. Finally the cells were rinsed, dehydrated and embedded in Epon 812 (TAAB, England) following a rapid time-schedule (Chang, 1972). After a slow polymerization (1 night at 37°C, 1 day at 45°C and 3 nights at 60°C), blocks were cut in the Falcon flask and mounted on a LKB III ultramicrotome; the plastic was peeled off with a scalpel, thin sections (400 Å) were directly cut with a diamond knife in the plane of the plastic substrate, picked up on copper grids, stained with uranyl acetate and lead citrate, and viewed in a Philips EM-301 microscope operated at 60 kV. The source of chemicals used was Merck (Darmstadt, Germany), except where otherwise specified.

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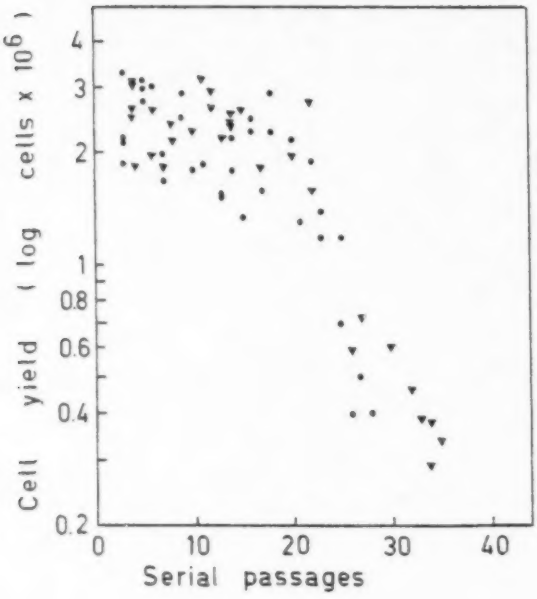
Inactivation curves of glucose-6-phosphate dehydrogenase

Cells were cultivated in Roller bottles. When confluent, the cells were rinsed twice with 10 ml of phosphate buffered saline (PBS) collected with a rubber policeman in 50 mM TRIS-HCl (pH 8) containing 10^{-3} M mercaptoethanol, 10^{-3} M EDTA, 4×10^{-3} M ε-amino-n-caproic acid, 3×10^{-3} M NADP and 1 mg of BSA per ml. Cells were homogenized and a supernatant fraction obtained (Remacle *et al.*, 1980). The inactivation curves were obtained by incubating the supernatant at 59°C. At given times, samples were removed and the residual activity tested by methods already described (Holliday and Tarrant, 1972; Houben and Remacle, 1978). The source of chemicals used in the enzyme assay was Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

Cultures from four different litters were started, called strains HP I, II, III and IV. They were subcultivated in exactly the same way; for strains HP I, II and IV the *in vitro* lifespan was approximately the same, but shorter than observed for strain III. So, for convenience sake, we will give the results for strains III and IV.

When a fully sheeted primary culture was obtained, cells were serially subcultivated; the cell culture rate, expressed as divisions per day varied around a value of 0.35 during the first part of the culture and then decreased at culture passages 20 (HP IV) and 25 (HP III). Concomitantly, as shown in Fig. 1A, the number of cells at saturation density followed the same evolution with time in culture: first it was stabilized between 2–3 × 10^6 cells/25 cm^2 for about 20–25 passages and then the saturation density decreased drastically in the last stages. Besides, cells while attaching to their substrate never succeeded in achieving another population doubling, even when changing the medium. This was the case for the non-frozen cells as well as for the frozen cells and occurred invariably at passage 27 for strain HP IV and 34 for strain HP III (Fig. 1A). Another expression of the results is given in Fig. 1B which showed that after a period of exponential growth referred to as phase II by Hayflick and Moorhead, 1961, cells entered a period of declined growth, phase III, which becomes obvious at culture passage 20 for strain HP IV and 25 for strain HP III. It is interesting to stress here that for strains HP I, II and IV the donor embryos were 14–15-days-old, whereas they were only 12-days-old in the HP III strain.



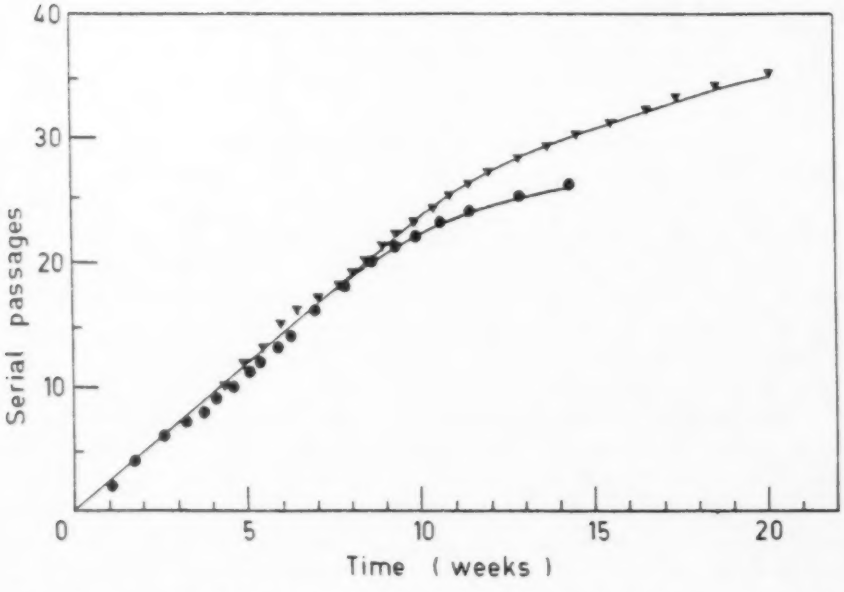


FIG. 1. Growth of HP cells in culture. HP cells were collected from hamster embryos and regularly cultivated as described in materials and methods. (A) Number of cells per plate (25 cm^2) along the cell culture (▼) Strain HP III and (●) strain HP IV. (B) Evolution of the culture with time. (▼) Strain HP III and (●) strain HP IV.

The morphology of the cells was routinely followed at phase II and III of the culture with a phase contrast microscope. In phase II cultures, cells were predominantly fusiform, or tripolar, with a small ovoid nucleus (Fig. 2, A1 and A2). These cells will be referred to as type 1 fibroblasts. In the same culture, we could also notice the presence of some few larger, more flattened cells; with culture passages, these large flattened cells became more and more numerous, they lost the typical fibroblastic shape and polarity, acquired distorted outlines and very often showed two nuclei (Fig. 2, B1 and B2). They will be referred to as type 2 fibroblasts in agreement with the designation of Franks and Wilson, 1977.

To understand the decline of the culture growth potential more clearly, we measured the thymidine incorporation of cells at different culture passages as described by Cristo-

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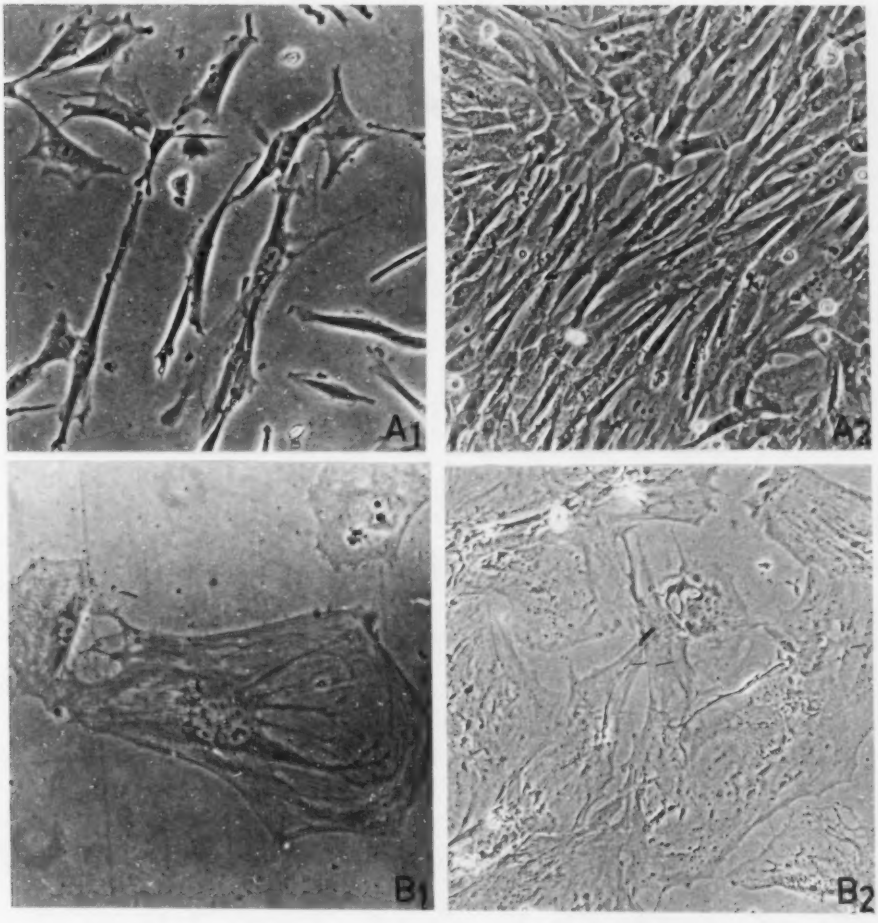

FIG. 2. Phase contrast micrographs of HP cells. (A) Type 1 fibroblasts observed during phase II of the culture when non confluent (A_1) or confluent (A_2). Note the numerous mitoses, × 128. (B) Type 2 fibroblasts observed during the last stages of phase III in non confluent (B_1) or confluent cultures (B_2). Note the lobulated nuclei, × 128.

falo and Sharf (1973). Considering the increasing morphological heterogeneity, this determination was carried out separately for the type 1 and type 2 fibroblasts (Fig. 3). The incorporation levels were maintained around values of 90%, 55% and 88%, respectively for the type 1, type 2 and the total cell population, until the life-spans completed (LSC) of the cells were 0.5 (HP IV) and 0.7 (HP III). Afterwards, the proportion of unlabelled cells increases linearly (in a semi-logarithmic scale) particularly for the type 1 fibroblasts; the latter thus seem to be mainly responsible for the general ageing behaviour of the cell population.

To have a better insight into the individual capacity of cell division, cloning was performed at different PDLs. Experiments were performed only on cells from strain III. After a 10-day incubation clones were observed, and their size and phenotype reported. Clone sizes were generally expressed as the total number of generations achieved. As shown in Fig. 4, the cumulative percentages of dividing clones reported to clone size followed a bimodal curve for cells taken from young cultures and an exponential-like curve when taken from old ones. Similar behaviour was observed on human fibroblasts (Smith and Hayflick, 1974; Martin *et al.*, 1975). The decrease in the division potential with ageing

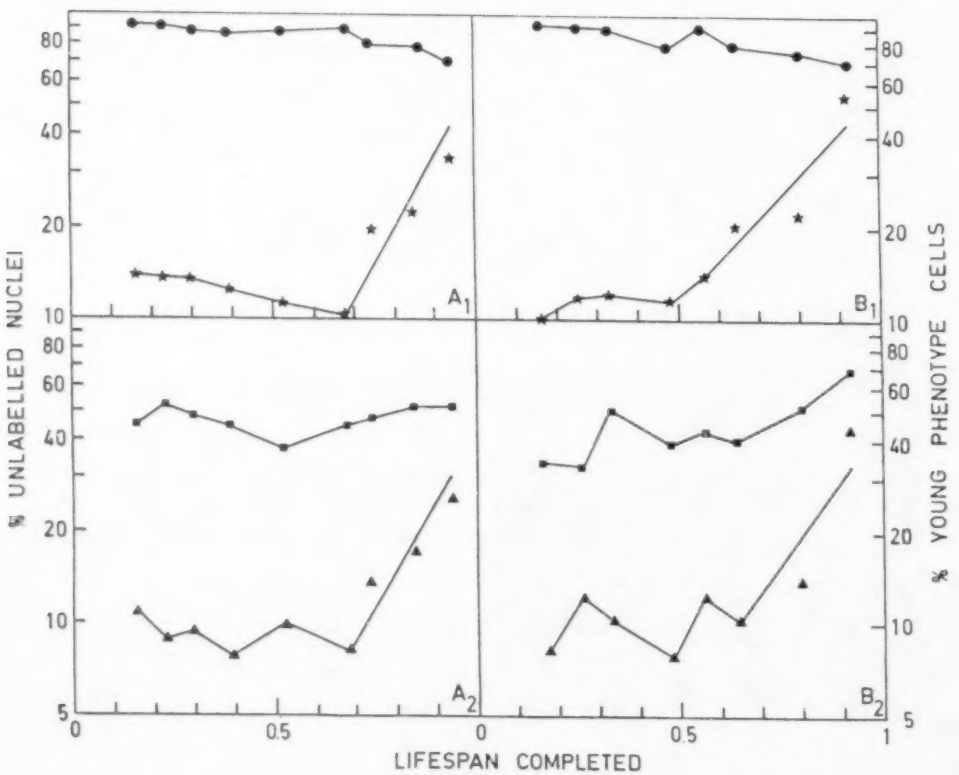

FIG. 3. Evolution of the labelling index in HP cell cultures expressed as the percentage of unlabelled nuclei in the overall culture (★) in type 1 (▲) and type 2 fibroblasts (■). Also (●) percentage of type 1 fibroblasts. (A) Strain III and (B) strain IV.

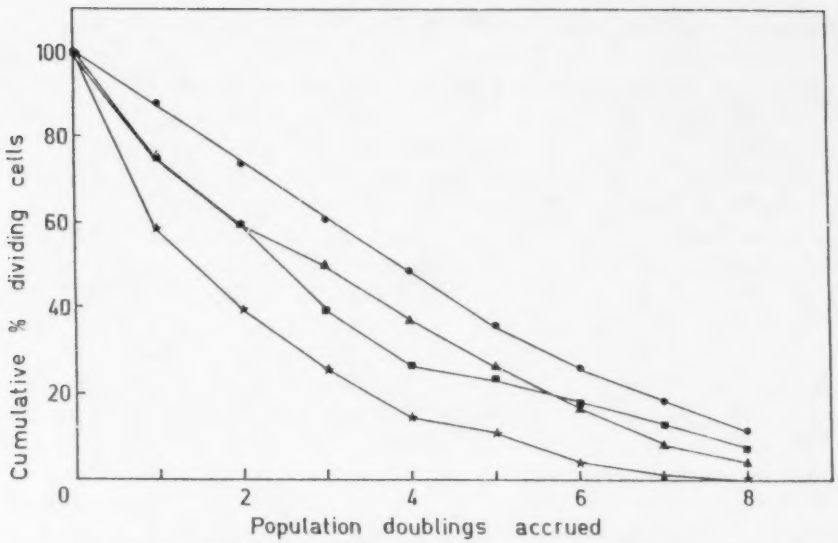

FIG. 4. Percentage of isolated cells undergoing a specific number of doublings with cell ageing. Four sets of clones isolated from cultures with a LSC of 0.25 (●), 0.48 (■), 0.65 (▲) and 0.75 (★) were cultivated as described in materials and methods. The results give the number of doublings achieved by each dividing cell of the clone, expressed in a cumulative manner.

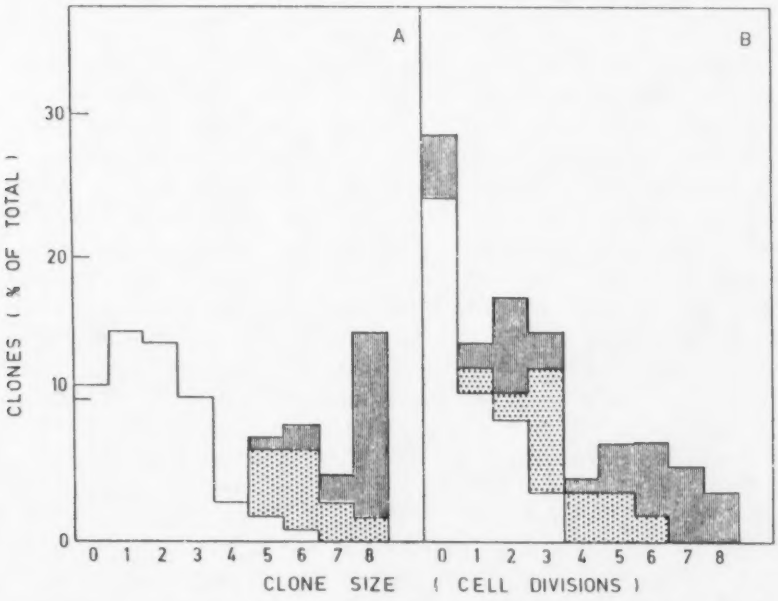

FIG. 5. Distribution of cell doublings achieved by cloned fibroblasts isolated from cultures with a LSC of 0.25 (A) and 0.65 (B). Clones were classified according to their sizes. Following the morphology of the cells, they were also considered as type 1 (hachured) or type 2 (clear). When no phenotype was dominant, clones were considered as intermediary (stippled).

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can be exemplified: first, by the fact that 50% of the cells reached at least 4 generations when taken from a culture with a LSC of 0.24, only 3 when their LSC was 0.64 and less than 2 when their LSC was 0.76; second, the maximum number of generations achieved clearly declined, ranging from more than 8 for a LSC of 0.24 to 6 for a LSC of 0.76. We also took into account the phenotype of the cells: clones were considered as type 1 when mainly composed of type 1 fibroblasts, as type 2 when mainly composed of type 2 fibroblasts, and as intermediary when both phenotypes coexisted.

Fig. 5A illustrates that cells cloned from an early culture, with a LSC of 0.24, produced clones that could be classified into two groups: in the first one, clones formed mainly by type 2 fibroblasts achieved a very limited capacity of division; in the second group, cells divided more than 4 times during the 10 days experiment and the corresponding clones contained mainly type 1 cells (Fig. 6A), or a mixture of both phenotypes then classified as intermediary clones (Fig. 6B). When cells were cloned from a later culture with a LSC of 0.65 (Fig. 5B), clones could also be classified in type 1 and 2, but the distributions of both groups were superimposed since type 1 and intermediary clones achieved a much lower division potential. When the phenotype of the cells was examined within the clones along the experiment, some modifications in the phenotype could be observed. For example, some clones composed of type 1 cells in the first divisions gradually acquired the type 2 phenotype and these type 2 cells accumulated mainly at the clone periphery. Such a clone classified as intermediary is depicted in Fig. 6B. This intraclonal differentiation process probably explains why 70% of the clones were recorded as type 2 in Fig. 5A, whereas the type 2 cells represent only 10% in the mother culture. Cloning efficiency varied between 5 to 15% and drastically declined when the LSC of the culture reached 0.8.

The examination of HP cells by TEM at different stages of culture confirmed the existence of two distinct phenotypes. In young cultures where type 1 cells are predominant (Fig. 7) most cells have an ovoid nucleus, with fine granular chromatin distributed homogeneously throughout the nuclear matrix. Generally condensed chromatin is concentrated along the nuclear membrane (not shown). In the cytoplasm, numerous elongated mitochondria, seem particularly well preserved with parallel cristae traversing the width of the organelle. Some mitochondria are closely associated or even encircled with endoplasmic reticulum portions. Some lysosomes and autophagic vacuoles, containing laminated structures are scattered throughout the cytoplasm. The endoplasmic reticulum of type 1 cells is well developed with moderately swollen cisternae and a more abundant rough component. Free ribosomes are numerous and organized in rosettes. The Golgi system is recognizable. Pinocytotic vacuoles originating from the cell membrane are often seen at the cell periphery. The type 2 fibroblasts more frequently seen in very old cultures have the following morphological features (Fig. 8): nuclei are generally lobulated and irregularly shaped with the chromatin distributed heterogeneously throughout the nuclear matrix. Nuclear bodies are often present. Mitochondria are numerous, but show a reduced structural integrity; they are generally shorter and more rounded up than in the type 1 cells and their association with RER portions seems less defined. Lysosome-like dense bodies, containing amorphous material, cytoplasmic debris are frequent. The ER appears extremely heterogeneous: in some cytoplasmic areas, the cisternae are distended with fine granular material whilst in others, they are narrow. Besides the smooth component is more evident than in the type 1 cells. Concomitantly with the ER degranulation, there are many free unclustered cytoplasmic ribosomes (monosomes). The Golgi system seems particularly well developed, with many vesicles and some swollen saccules. Pinocytotic activ-

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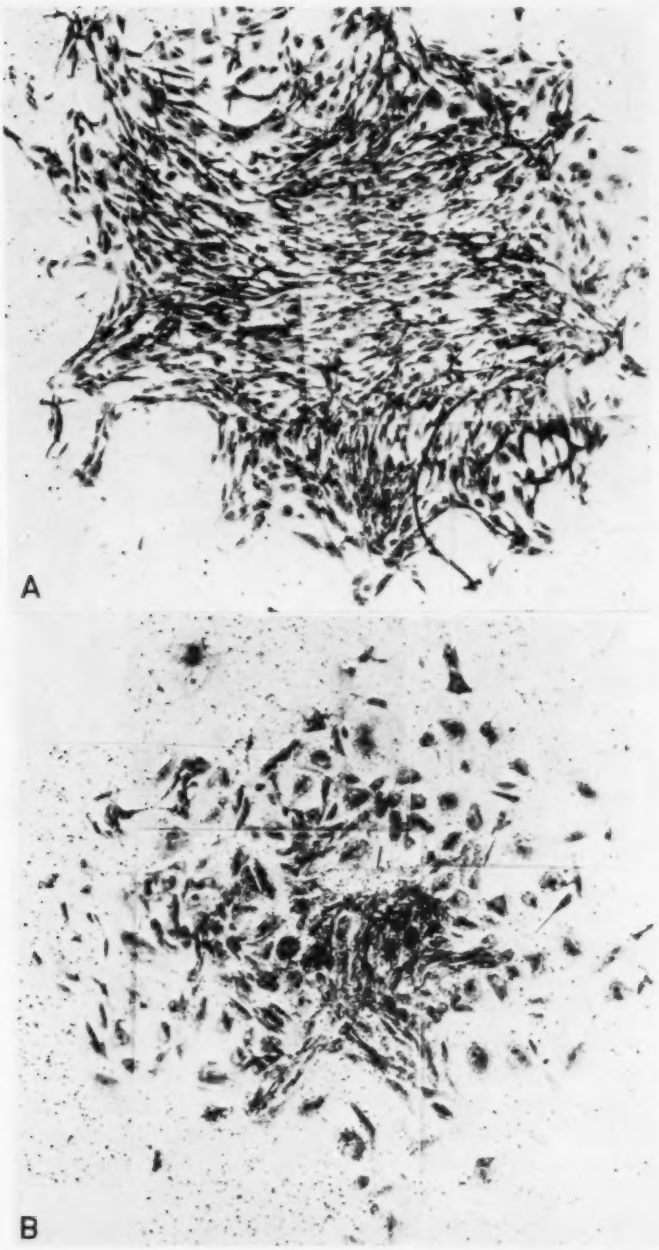

FIG. 6. Micrographs of clones obtained from two cells isolated from a young culture (LSC 0.24). In A, the number of divisions is high; cells are of type 1 and keep the regular arrangement typical of a young culture. Notice however, the appearance of some bigger cells at the periphery (arrows). In B, the number of divisions is lower, the clone contains type 1 cells in its center and type 2 cells at the periphery (arrows); this clone was classified as intermediary clone. May Grunwald Giemsa staining, × 73.

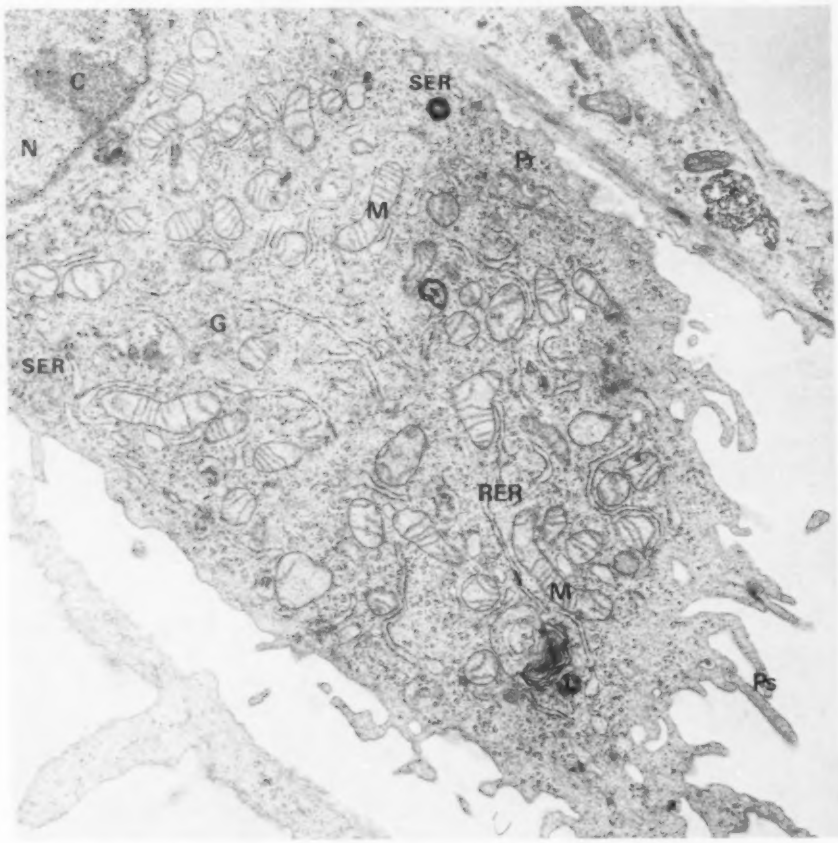

FIG. 7. Electron micrograph of type 1 fibroblasts from a young culture. The LSC of the cell culture was 0.4. Note the well preserved structure of all the subcellular organelles. N. nucleus, C. condensed chromatin, M. mitochondria, RER. rough endoplasmic reticulum, SER. smooth endoplasmic reticulum, G. golgi, L. lysosomes, Pr. polyribosomes, Ps. pseudopodia, × 12,150.

ity was also observed (not shown here). The ultrastructural observations of cytoskeleton are given in the following paper (Raes *et al.*, 1983).

With the ageing of fibroblasts *in vitro*, a lot of enzymatic changes have been described. One is the appearance of modified enzymes during the ageing of cell cultures. For instance the presence of a more thermolabile fraction for the glucose-6-phosphate dehydrogenase has been described in aged human fibroblasts (Holliday and Tarrant, 1972; Houben and Remacle, 1978). We investigated the possible presence of an altered fraction of this enzyme on a supernatant fraction from young (LSC of 0.25) and old cells (LSC of 0.85). The thermolability curves are given in Fig. 9. In young cultures, the experimental values could be drawn by a regression line with no significant deviation from linearity. In old cultures, the experimental values fitted best with the superposition of two decreasing exponentials: the first, with the steeper slope gives the denaturation rate of the heat-labile fraction of the enzyme; the second, that of the unaltered enzyme. The latter was responsible for 80% of the total enzyme activity.

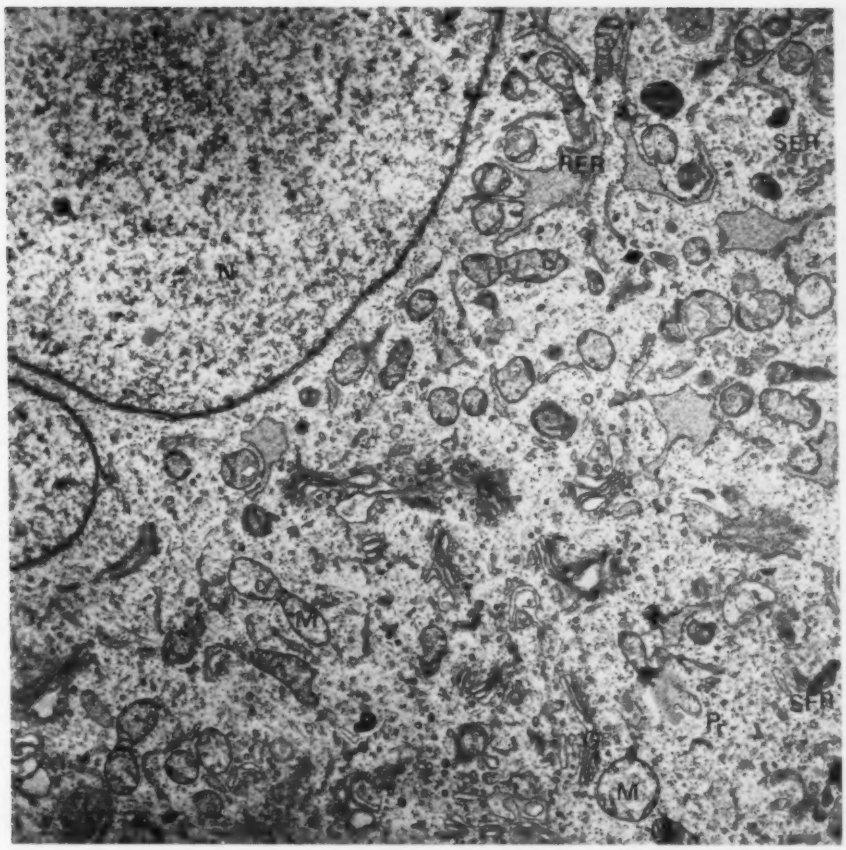

FIG. 8. Electron micrograph of type 2 fibroblasts form an old culture. The LSC of the cell culture was 0.95. Note the deterioration of the structure mainly observed in the heterogeneous distribution of chromatin (C), the low density of structures in the cytoplasm especially of polyribosomes (Pr) and the loss of mitochondrial cristae (c). N. nucleus, M. mitochondria, RER. rough endoplasmic reticulum, SER. smooth endoplasmic reticulum, G. golgi region, L. lysosomes, × 12,150.

DISCUSSION

WI-38, MRC-5, IMR-90 and TIG-1 human fibroblasts are cell strains universally used in the study of ageing. In this work we wanted to start cultures of embryo lung fibroblasts from another species in order to understand the phenomenon of ageing in mammals more clearly. The golden hamster (*Mesocricetus auratus*) was chosen for practical reasons and for its short maximum life-span of 2–3 years (Bruce and Hindle, 1934).

When cultured, the hamster fibroblasts went *in vitro* through an exponential phase of growth ending with the senescence phase already described for human fibroblasts (Hayflick and Moorhead, 1961). The maximum PDL reached by our cells was 34. Although we did not find any data on serially subcultivated hamster embryo lung fibroblasts, these results are in agreement with cultures of other rodent fibroblasts, which show a similar senescence phase generally followed by spontaneous transformation when obtained from rat embryos (Meek *et al.*, 1980) and from BALB/c mouse embryos (Meek *et al.*, 1977).

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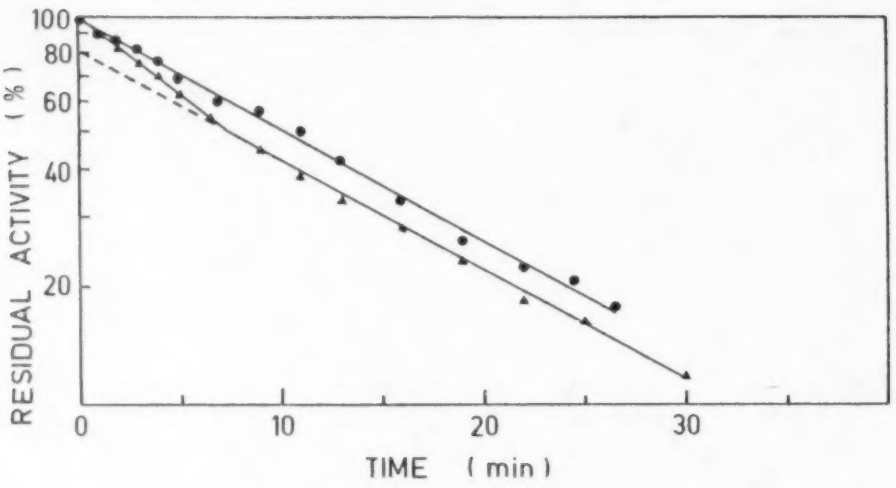

FIG. 9. Thermolability of G6PD from young (●) and old (▲) HP fibroblasts. HP cells with a LSC of 0.30 considered as young cells and 0.90 considered as old cells were homogenized and the supernatant prepared as described in materials and methods. The inactivation curve was performed at 59°C.

Unexpectedly, we observed no such transformation but recorded high reproducibility in the evolution of the cultures which end up with approximately the same maximum *in vitro* life-span although derived from 4 different litters. Interestingly, the strain with the highest division potential was precisely the one derived from the youngest embryos. Although we are aware that these data are not sufficient to be conclusive, this higher *in vitro* division potential of the cells could be the result of their shorter *in vivo* past. However, Hay, 1970 reported no difference in the division potential of fibroblast cultures from 12 and 16 days-old-mice embryos.

With the *in vitro* ageing, we observed an increasing heterogeneity in the cell culture with the appearance of 2 main phenotypes: type 1 phenotype, fibroblast-like cell, fusiform or tripolar, with an ovoid nucleus; and type 2 phenotype, non-fibroblast-like cell, large, flat, with one or more nuclei and numerous slender processes. Morphological heterogeneity in fibroblast-like cultures has been described for WI-38 fibroblasts (Wolosewick and Porter, 1976) and for rat fibroblasts (Kontermann and Bayreuther, 1979). For these authors the morphological change is associated with the differentiation of rat fibroblasts into fibrocytes. Besides, increasing cell size has been associated with the *in vitro* ageing, of human fibroblasts (Macieira-Coelho and Ponten, 1969; Cristofalo and Kritchevsky, 1969; Macieira-Coelho, 1970; Schneider and Fowlkes, 1976; Greenberg *et al.*, 1977; Matsumura *et al.*, 1979; Houghton and Stidworthy, 1979), marsupial fibroblasts (Simons, 1970), mice embryo fibroblasts before spontaneous transformation (Tuffery and Baker, 1973; Van Gansen *et al.*, 1979), rat embryo fibroblasts (Kontermann and Bayreuther, 1979) and chicken embryo fibroblasts (Lima and Macieira-Coelho, 1972). However, in none of these cases, was the change as dramatic as the one described here.

The ultrastructure of *in vitro* ageing cells has been described by many authors: Robbins *et al.*, 1970; Lipetz and Cristofalo, 1972; Johnson, 1979 studied human embryo fibroblasts; Brunk *et al.*, 1973, human embryo glial cells; Brock and Hay, 1971, chicken em-

bryo cells; and Van Gansen *et al.*, 1979, mouse embryo cells. However, these data although roughly concordant, are somewhat contradictory. Franks and Wilson reviewed the problem, 1977 and they observed two main cell types that they called type-1 and type-2 cells. It is because of the strong similarities between the morphology of these two cell types and the HP fibroblasts described here that we used the same designation. We are aware that structure variations may be related to the growth phase of cells as pointed out by Comings and Okada (1970), but all HP cells examined were always preconfluent. Thus in agreement with previous observations on other cell types, the characterization of the type 2 fibroblasts (Fig. 8) are as follows: a lobulated and irregular nucleus with a more condensed chromatin than in the type 1, altered mitochondria, marked degranulation of RER with some extremely swollen cisternae, many unorganized free ribosomes, hypertrophied and vesiculated Golgi system and the accumulation of lysosome-like dense bodies. These morphological observations must be relevant of some physiological changes in these type 2 cells. Chromatin condensation is known to be related to a reduced genetic activity. Concomitantly, free unclustered ribosomes (monosomes) may be significant of a reduced protein synthesis. Interestingly, Comings and Okada, 1970 on human fibroblasts and Goldberg and Green, 1964 on 3T6 mouse fibroblasts, observed the same features in cells at confluence, thus with a reduced metabolic activity. Besides, the reduced structural integrity of mitochondria, even if resulting from a higher sensitivity to fixation and embedding procedures, must be significant of functional alterations that could drastically impair cell survival and division. The development of the Golgi system, also pointed out by Lipetz and Cristofalo, 1972 on WI-38 fibroblasts, could be explained by the implication of this system in primary lysosome formation. These primary lysosomes could later differentiate in lysosomal dense bodies and autophagic vacuoles. Less evident is the dilatation of RER cisternae in type 2 cells and data on this point are rather contradictory. However, swollen cisternae were also observed in stationary confluent cells (Comings and Okada, 1970; Goldberg and Green, 1964). Some of these modifications are the result of detrimental physiological functions which impaired the cell dynamism and division, others probably result from the low rate of division of these type 2 cells and their long stationary time in culture. However the ultrastructure of HP cells also reveals the two distinct phenotypes.

A biochemical criterion of ageing was also searched. We were interested by the possible presence of altered enzyme in ageing cultures (Holliday and Tarrant, 1972) and more particularly with G6PD, already studied in our laboratory (Houben and Remacle, 1978; Houben, 1981); we found no altered enzyme in young cultures but a small amount in old ones (Fig. 9). Although the presence and the mechanisms for altered G6PD in ageing cultures or tissues is quite controversial (Yagil, 1976; Pendergrass *et al.*, 1976; Schofield and Hadfield, 1978)—the heterogeneity of the systems used probably explains part of this controversy—we provide evidence for an effective but small alteration of G6PD in an *in vitro* ageing rodent cell culture. This corroborates the results of Wulf and Cutler, 1975 on ageing mice tissues.

The ageing of cells especially their increasing cell size along the evolution of the culture has been associated with a decreased DNA synthetic ability (Bell *et al.*, 1978; Bowman *et al.*, 1975; Absher and Absher, 1976; Mitsui and Schneider, 1976 *a, b*). The question raised by these authors was whether the dynamism of the cell population or part of it was affected by the modification of the phenotype. Most of them notice that the mitotic cells remain small and maintain their high DNA synthetic ability, at least during the exponen-

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tial phase of the culture. We approached this problem, by measuring the thymidine incorporation in old and young cultures, discriminating between the two phenotypes (Fig. 3). In agreement with Vincent and Huang (1976), we observed that the labelling index was stable during ⅔ of the culture. The plateau values were 90, 55, and 88%, respectively for type 1, type 2 and for the total cell populations. When 70% of the life-span was completed, the proportion of type 1 cells as well as the labelling index decreased drastically for type 1 cells and for the total cell population, but slowly for the type 2 cells. These results are in disagreement with the report of Cristofalo and Sharf, 1973, which showed a decreasing labelling index early in the culture of WI-38 fibroblasts. Anyway, the presence of a plateau seems quite acceptable to us because of its correspondence with phase II, while the beginning of phase III corresponds with a decrease in the labelling index. Since the type 1 cells have a higher DNA synthetic ability than the type 2 ones and since their proportion remains constant during phase II (about 90%), we conclude that some of them continuously transform into type 2 cells. It is possible to calculate from our data the percentage of type 1 cells that differentiate into type 2 ones at each subdivision and during a two day period. For these calculations, we postulate that cells non-labelled during the 24 h incubation time are not definitively unable to divide; this assumption is in agreement with many former data (Bell *et al.*, 1978; Absher and Absher, 1976; Macieira-Coelho, 1974).

Results of these calculations are reported in Fig. 10. They show that a given proportion of cells, about 2%, differentiate at each subcultivation and in 48 h during the exponential growth of the culture (phase II); at the beginning of phase III this proportion significantly increases and this modification is concomitant with the decrease in the division rate observed in type 1 cells (Figs. 1 and 3).

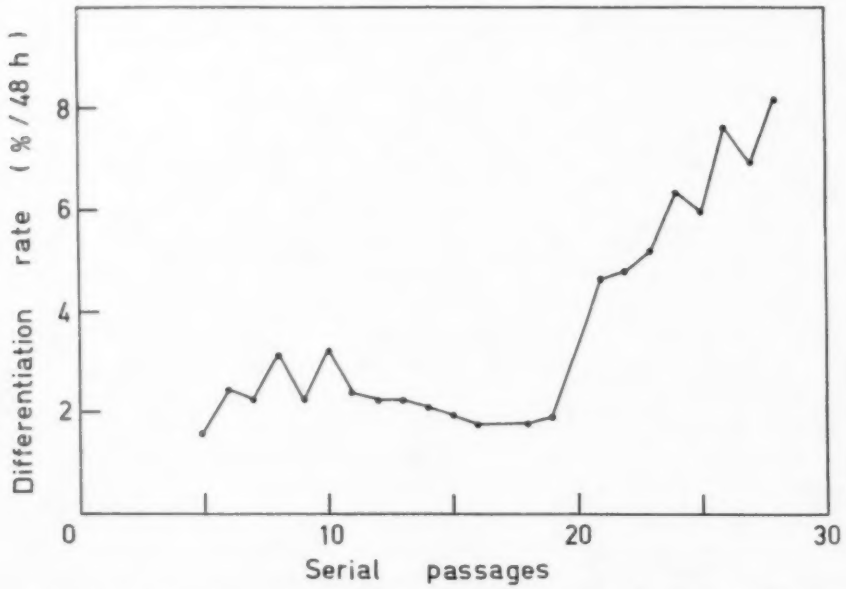

FIG. 10. Rate of differentiation of HP type 1 fibroblasts into type 2 fibroblasts. Results are calculated from data presented in Figs. 1 and 3A and give the % of type 1 cells which differentiate in 48 h with the passage number of the culture.

This differentiation process could also be individualized in the cloning experiments since type 2 cells progressively accumulated in clones originally composed of young phenotype cells (Fig. 6). The bimodal distributions of clones obtained from young culture cells (Figs. 4 and 5) can be explained by the presence of two populations: one containing type 2 cells with a low division potential and another one with actively proliferative type 1 cells which then partly differentiate in the last divisions. The cloning experiments are thus in agreement with the observations on mass cultures despite the limitations imposed notably by the low cloning efficiency.

One question raised by these observations is whether the differentiation of the cells giving rise to type 2 cells with a low division capacity can explain the ageing of the cells *in vitro*? This does not seem to be the case. Indeed Fig. 3 clearly shows that if the number of type 2 cells increases with age, the amount of type 1 cells is still important in old cultures but their division rate slows down spectacularly in the last passages. Thus, these type 1 cells appear to undergo at the end of the culture a fast ageing process besides the differentiation process affecting an increasing proportion of them. Also in the cloning experiments on cells from a late culture (Fig. 5B), the division capacity of type 1 cells decreased, compared to the same cells originating from a young culture (Fig. 5A); some of these cells didn't even have the possibility to divide. Thus, also in the cloning experiment, type 1 cells reach a limit in their division capacity. Phase III which starts when cells have completed around 70% of their maximum life-span, clearly results from both a decrease in the division rate of the cells and from an increase in the differentiation rate (Figs. 3 and 10). It seems at this point that type 1 cells are modified in such a way that they begin to age and to differentiate in an increasing, irreversible way.

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One problem raised by the observation of cells in culture is whether ageing is the result of a differentiation process or if it is a stochastic event influenced by other extrinsic variables? There is disagreement on this matter. Some authors effectively observe during ageing in culture or in cloning experiments the appearance of phenotypical modifications suggesting a differentiation process of the cells (Bell *et al.*, 1978; Martin *et al.*, 1975; Kontermann and Bayreuther, 1979); others provide evidence for the high variability in the time and the potential of division in sister cells and explain more logically this variability by stochastic mechanisms (Shmookler-Reis *et al.*, 1980; Smith and Whitney, 1980; Absher and Absher, 1976). The same results can however be interpreted in different manners.

Because of their 2 distinct phenotypes, hamster fibroblasts allowed us to follow their evolution not only in cloning studies, but also in mass cultures. In this context, we first observed a differentiation process starting from the beginning of the cell culture and holding on during subcultivations and clonal experiments. Once differentiated, cells could perform only a few divisions before stopping. This process can exemplify the clonal attenuation described by Martin *et al.*, 1974. The factors which affect the differentiation rate of the cells are not yet known (Bell *et al.*, 1978). Parallel to this cellular differentiation, a cellular ageing process occurs affecting mainly type 1 cells late in the culture and characterized by an obvious lengthening of their cell cycle.

We thus believe that cells can differentiate in culture and end up as non-dividing type 2 cells but that besides this differentiation process there exists a so-called ageing process, which appears as a rapid phenomenon, consistent with the vicious circle of the catastrophe error (Orgel, 1973). Other theories like the commitment theory (Holliday *et al.*, 1977) or the "counting mechanism" (Good and Smith, 1974) cannot be excluded to explain this later phase. Growth arrest could thus result either from a cellular differentiation ge-

netically programmed or from any other stochastic phenomenon. The apparent contradictory results obtained in the literature could be explained if we accept the possibility of both mechanisms shown above as occurring simultaneously or independently depending on the cell type and the culture or experimental conditions used.

SUMMARY

Fibroblasts from hamster embryos were serially cultivated *in vitro* and their evolution was followed from a morphological, physiological and biochemical point of view. The cells were growing exponentially for about 20 passages before entering the ageing phase or phase III which ended up after 29–34 passages. Many structural and biochemical modifications were associated with this ageing process.

Along with the ageing process was associated a differentiation process, characterized by the appearance of large flat cells with a very low division potential called type 2 fibroblasts. The proportion of these cells was low up to phase III. Then their proportion increased due both to the increase in the differentiation rate of the small type 1 fibroblasts and the lowering of their division rate.

Cloning experiments confirmed the mass culture observations. They showed both the process of differentiation and ageing of the fibroblasts within the clones. Also clones obtained from old cell cultures contained some type 1 fibroblasts which had completed their division potential without being differentiated. The conclusions of these observations are that the arrest of growth results for some HP fibroblasts from a terminal differentiation mechanism and for others from a stochastic ageing process.

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MICROTUBULES AND MICROFILAMENTS IN AGEING HAMSTER EMBRYO FIBROBLASTS *IN VITRO*

M. Raes, G. Geuens*, M. de Brabander* and J. Remacle

Laboratoire de Biochimie Cellulaire, Facultés Universitaires, B-5000 Namur, Belgium
*Laboratory of Oncology, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse, Belgium

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Abstract—Microtubules and microfilaments were investigated in hamster lung fibroblasts, during their *in vitro* life-span. These cells show a senescence process characterized by a drastic phenotypic change, resulting in two phenotypes: the type 1 cells, characteristic of young cultures and the type 2 cells appearing progressively with culture passages. Microtubules and microfilaments were observed at the TEM and also vizualized by the unlabelled peroxidase-anti-peroxidase method. Moreover, the susceptibility of microtubules to nocodazole was tested in type 1 and 2 cells. We could not provide evidence for a different susceptibility to the drug. However the depolymerization wave occurred centripetally in type 1 cells whilst centrifugally in type 2 cells. These observations are discussed in relationship with the early arrest of division growth of the type 2 differentiated cells.

INTRODUCTION

Cytoskeleton components as well as being ubiquitous in the cell, are associated with many cellular functions such as cytokinesis, intracellular movements, cell motion, cell shape, . . . (for a review see Dustin, 1978). In the last 10 years, cytochemical techniques have been developed and improved in many laboratories working with different cell types, in order to visualize the cytoskeleton components. A large amount of this work has been carried out on microtubules, using techniques like, the unlabelled peroxidase-anti-peroxidase method (PAP) (De Brabander *et al.*, 1977; Henderson and Weber, 1979), immunofluorescence (for a review, see Brinkley *et al.*, 1980) and more recently the promising immuno gold staining method (De Mey *et al.*, 1981). All these authors observe a well defined cytoplasmic microtubule complex. Microfilaments have also been studied by the PAP method (Henderson and Weber, 1979), by immunofluorescence with anti-actin antibody (Lazarides and Weber, 1974; Heggenes *et al.*, 1977), by microinjection of labelled probes (Taylor and Wang, 1980) or using specific fluorescent stains (Barak *et al.*, 1981; Verderame *et al.*, 1980).

These techniques were also used to study the dynamism of the cytoskeleton: Osborn and Weber, 1976 studied the expression of microtubules during the spreading of mouse 3T3 cells and Badley *et al.*, 1980 observed the cytoskeleton changes in fibroblast adhesion

Correspondence should be addressed to Dr. J. Remacle.

and detachment; Ostlund *et al.*, 1980 provided some data on the microtubule regulation in cultured fibroblasts, observing that the percentage of tubulin assembled into microtubules increased in confluent cultures; many comparative studies in normal and their transformed counterpart cells have been carried out (for a review, see Brinkley *et al.*, 1980), but there is still some disagreement as to whether microtubule networks are altered or not in the transformed cells. Many inhibitors were also tested and their influence on the cytoskeleton investigated: for instance, the effects of sodium azide on microtubules (De Brabander *et al.*, 1981) and microfilament stability (Bershadsky *et al.*, 1980). Since the cytoskeleton is involved in so many essential functions which seem to be impaired during the ageing of the cells, we found it interesting to make a morphological as well as a physiological study of the cytoskeleton in hamster embryo lung fibroblasts. These cells show a senescence process *in vitro* characterized by drastic phenotypic changes (Raes and Remacle, 1983). In young cultures, typical fusiform fibroblast-like cells are mainly observed: we called them type 1 cells. With *in vitro* ageing, large, flat cells become more and more numerous: these were called type 2 cells. The differentiation process from type 1 into type 2 is followed by a very limited number of divisions before the cells stop growing. Until now, there is very little information about possible alterations of the cytoskeleton caused by ageing and when available this information produced only by electronmicrographs is quite incomplete (Van Gansen *et al.*, 1979). In this study, microtubules and microfilaments were investigated.

MATERIALS AND METHODS

Cell culture

Cultured lung fibroblasts (HP cells) were started from golden hamster (*Mesocricetus auratus*) embryos and subcultivated as described by Raes and Remacle, 1983. We used the strain HP IV described by these authors because it showed a typical senescence process. Other details concerning the modifications associated with the differentiation and ageing processes are presented in their paper. For the immunocytochemical procedure, cells were seeded on sterile coverslips (De Brabander *et al.*, 1977).

Transmission electron microscopy

Cells were fixed, dehydrated and embedded in Epon 812, *in situ*, as previously described (Raes and Remacle 1983).

Immunocytochemical staining

Coverslip cultures were fixed and stained for light microscopic immunochemistry, according to De Brabander *et al.*, 1977. HP cells were treated with nocodazole (R 17934) (Hoebeke *et al.*, 1976) at different concentrations (0.04, 0.1, 1.0 and 10 μg/ml) and for different times (15, 30, 60 and 240 min). We used affinity purified antibodies against dog brain tubulin and chicken gizzard actin.

RESULTS

We first investigated the cytoskeleton in type 1 and 2 cells, using the unlabelled peroxidase-anti-peroxidase method (Figs. 1 and 2). In type 1 phenotype cells, microtubules and microfilaments are present in a rather homogeneous arrangement. The labelled microtubules form a pattern of unbranched filaments that radiate in the perinuclear region from 1 or 2 points near the nucleus and are organized in the cytoplasm into parallel arrays, extending along the main cell axis and cell processes (Fig. 1a). Microfilaments appear like parallel arrays running along the axis of the cell; they turn

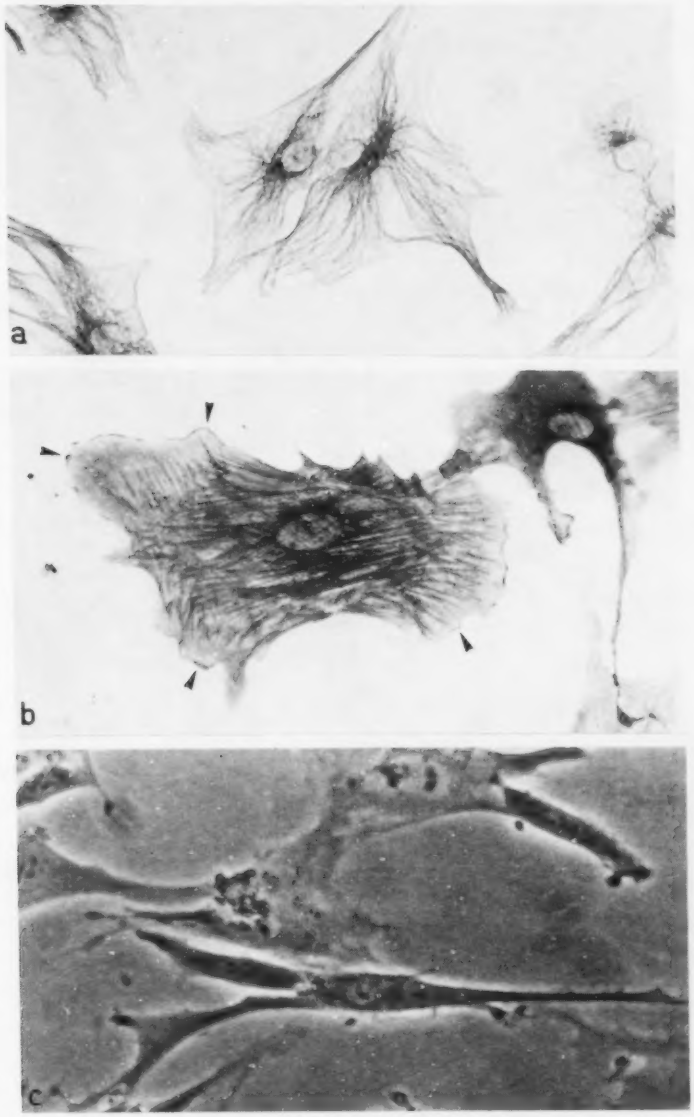

FIG. 1. Cytoskeleton in actively dividing type 1 HP cells. The life-span completed of the culture was 0.48. a. Microtubules observed after immunolabelling of tubulin. Note the well defined focal points, × 230. b. Microfilaments observed after immunolabelling of actin. Note the arrangement of microfilament bundles along the axial spreading of the cell and the accumulation of label at the edges of pseudopodia (arrows), × 230 c. Phase contrast micrograph of a typical type 1 cell, × 230.

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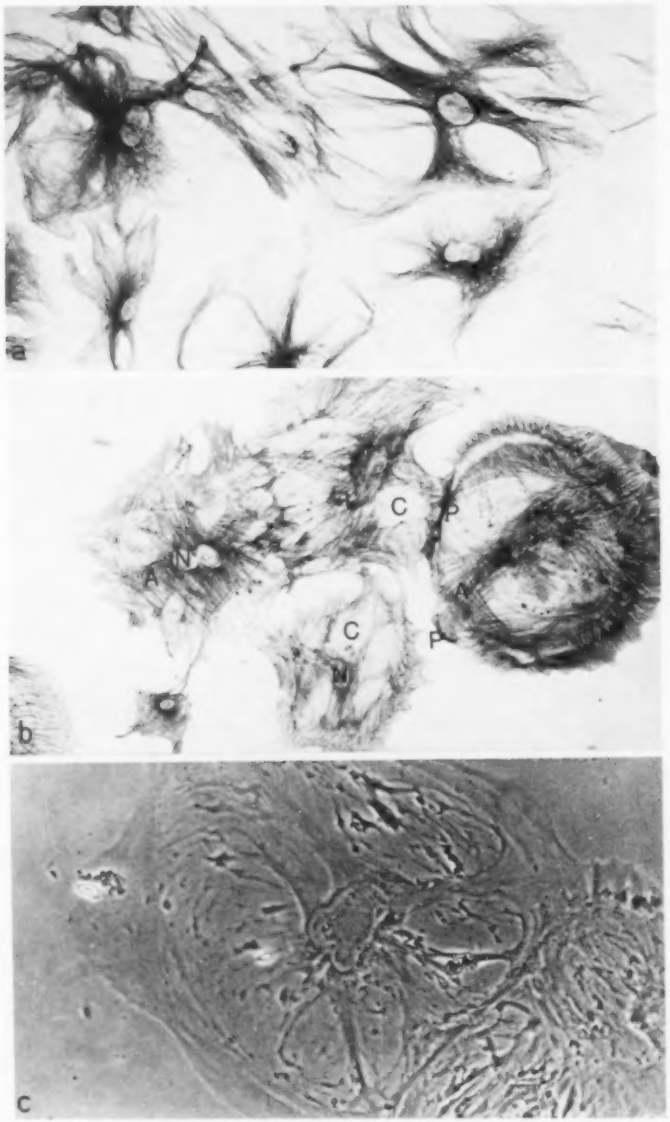

FIG. 2. Cytoskeleton in differentiated type 2 cells present in an old culture (life-span completed : 0.95). Pictures shown in a and b are given at a lower magnification than for the type 1 cells in Fig. 1a and b. a. Microtubules observed after immunolabelling of tubulin. Note the extensive network delimiting cytoplasmic areas devoid of tubules, × 92. b. Microfilaments observed after immunolabelling of actin. Note the heavily labelling of cell contacts (arrows). N : nucleus, C : cytoplasm with low actin content, P : actin patches, A : local accumulation of actin at the cell edges, × 92. c. Phase contrast micrograph of a typical type 2 cell. Note that the microtubule organization of fig. 2a is clearly seen in this picture, × 230.

aside from this main orientation in spreading cells, extending through pseudopodia, filopodia (Fig. 1b). They are particularly abundant at the cell to cell contacts and cell edges. In type 2 cells which are extremely large and flattened, peculiar and variable images can be seen: large bundles of microtubules start from the perinuclear region, delimiting other areas completely devoid of microtubules (Fig. 2a). The focal points in the perinuclear region are less defined in these cells. When reaching the cell cortex, part of these bundles end perpendicularly to the cell membrane; part of them bend, running parallel to the plasma membrane for long distances. As a consequence, microtubules from neighbouring bundles meet each other in the cell cortex. The microfilament patterns were constituted by large bundles crossing each other and extending radially to the cell periphery (Fig. 2b). However their distribution was much more heterogeneous than in type 1 cells: some areas appear heavily labelled as a result of the crossing over of several microfilament bundles; this is the case for the nuclear region and some cytoplasmic areas where heavily stained patches are often seen. On the contrary, other cytoplasmic areas are faintly stained with microfilament bundles having parallel orientation. In some large spread cells, 3 areas can be clearly distinguished: a cortical area, where short and heavily stained bundles extend to the cell periphery: a small annular region where very little polymerized actin is observed; and a central area where microfilament bundles cross each other as described above. Moreover, the contact between two neighbouring cells is always strongly decorated with microfilaments.

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We used a more dynamic approach to discover possible qualitative differences in tubulin and microtubules in the cells. Young and old cultures were treated with nocodazole a microtubule inhibitor (De Brabander *et al.*, 1976), at different concentrations and for different periods of time. For convenience and because of the similarity of the results, we will only consider the 4 hour treatments at increasing concentrations. In cells of type 1, at the lowest drug concentration (0.04 μg/ml, Fig. 3b) a partial depolymerization was observed in most cells, compared to the control cells (Fig. 3a). The depolymerization was however not uniform throughout the cell: the distal ends of microtubules were particularly affected, whilst an important core of stable microtubules radiating from the focal point(s) resisted the drug. At a concentration of 0.1 μg/ml (Fig. 3c), the depolymerization wave left a uniformly labelled cytoplasm, probably corresponding to free tubulin; the remaining polymerized portions of tubulin were concentrated at the focal point(s) near the nucleus. At higher concentrations (not shown), microtubules were completely depolymerized. In cells of type 2 (Fig. 4) after a 4 hour-incubation with nocodazole at 0.04 μg/ml (Fig. 4b), microtubules were partially depolymerized. Unexpectedly, the depolymerization wave started from the nuclear region to the cell periphery whereas free tubulin was redistributed more homogeneously, when compared to the control cells (Fig. 4a) in the affected part of the cytoplasm. At a concentration of 0.1 μg/ml, the depolymerization wave reached the cell cortex, however a core of resistant microtubules in the perinuclear region was often present. At higher concentrations, depolymerization was complete (not shown). Thus depolymerization was observed in both phenotypes at similar concentrations and for the same incubation time; we could not therefore provide evidence for a clearly different susceptibility to the drug with *in vitro* ageing. After nocodazole treatment, however, type 2 cells behaved unexpectedly, showing a centrifugal depolymerization wave compared to the centripetal wave in type 1 cells. As well as this, their anchorage to the focal points seemed less defined than in type 1 cells.

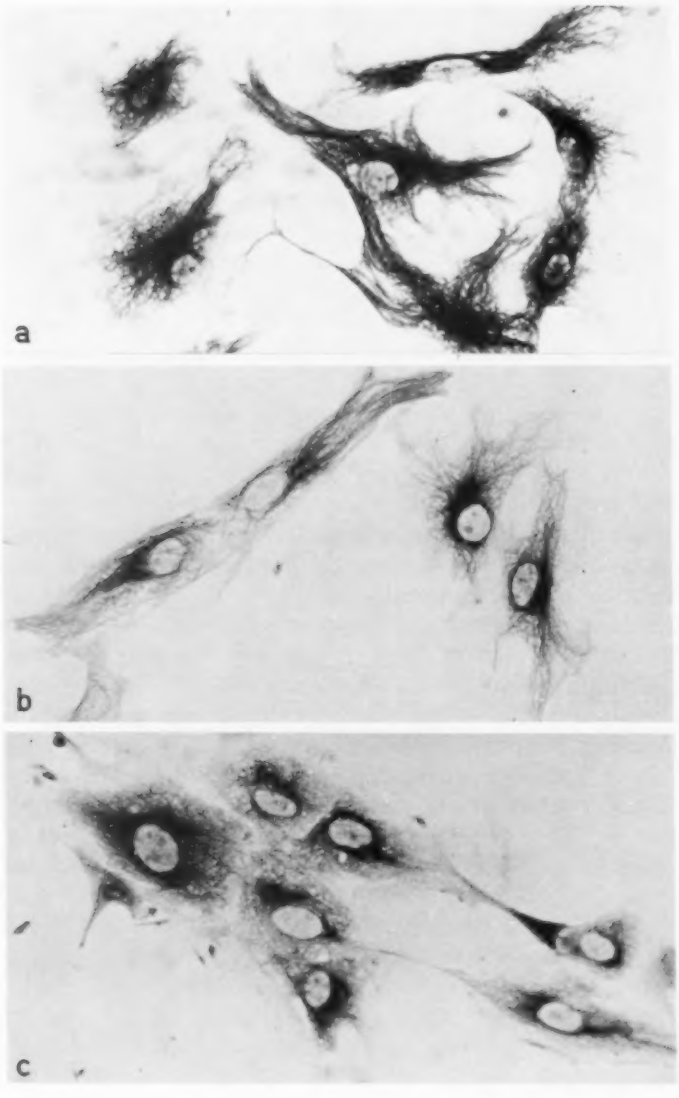

FIG. 3. Microtubules organization of type 1 HP cells before (a) and after treatment with 0.04 (b) and 0.1 μg/ml (c) of nocodazole for 4 h, × 230.

The TEM observations of HP cells provided some complementary data on the cytoskeleton, particularly in the cell cortex, this cytoplasmic area from which ribosomes and other organelles are excluded. In type 1 cells, the plasma membrane is sustained by a network of microfilaments, particularly developed at intercellular contacts as showed in Fig. 5. But in this network microfilaments apparently assume two kinds of configurations: first of all, the plasma membrane seems sustained by a 3-dimensional matrix of microfilaments with an amorphous aspect where individual microfilaments are inserted in

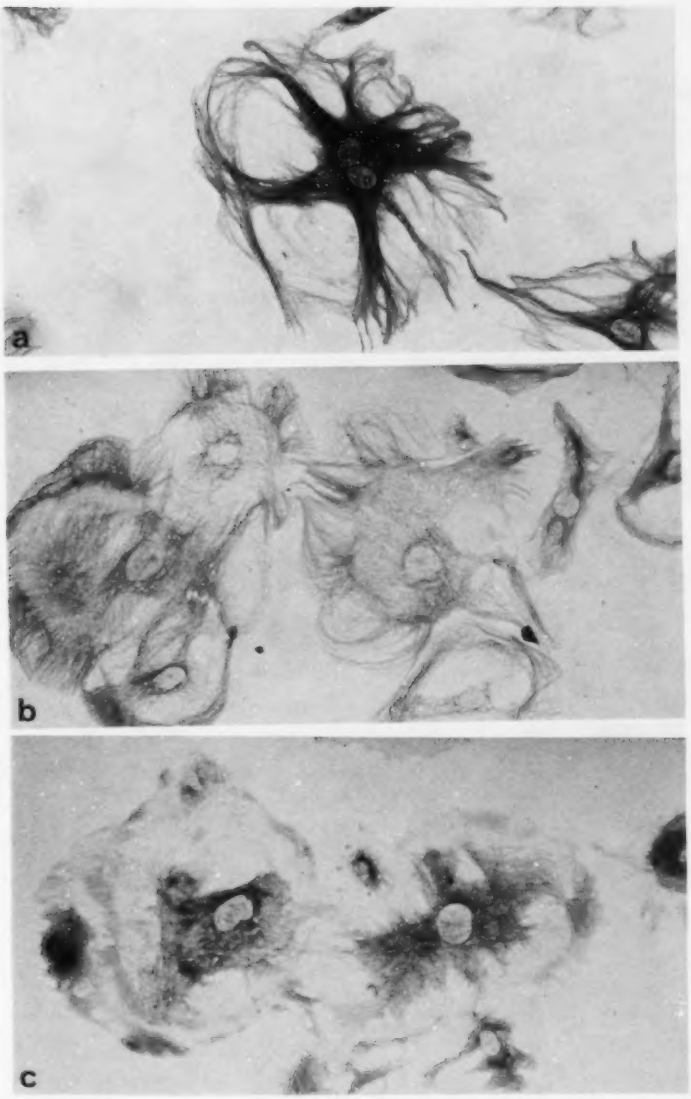

FIG. 4. Microtubules organization of type 2 HP cells before (a) and after treatment with 0.04 (b) and 0.1 μg/ml (c) of nocodazole for 4 h, × 92.

the matrix. The orientation of these bundles is variable. As shown in Fig. 5, one cell (A) has bundles clearly perpendicular to the cell edge, whereas in the other, they are rather parallel to the cell edge (B). Pinocytotic vesicles are often seen in this cortical layer. In the remaining cytoplasm, microfilaments are scarcely seen. Microtubules, although recognizable throughout the cytoplasm, also seem better preserved in the cell periphery. In the cortical layer, individual microtubules end in the microfilament bundles, following more or less accurately, the orientation given by the microfilaments. As shown in Fig. 5,

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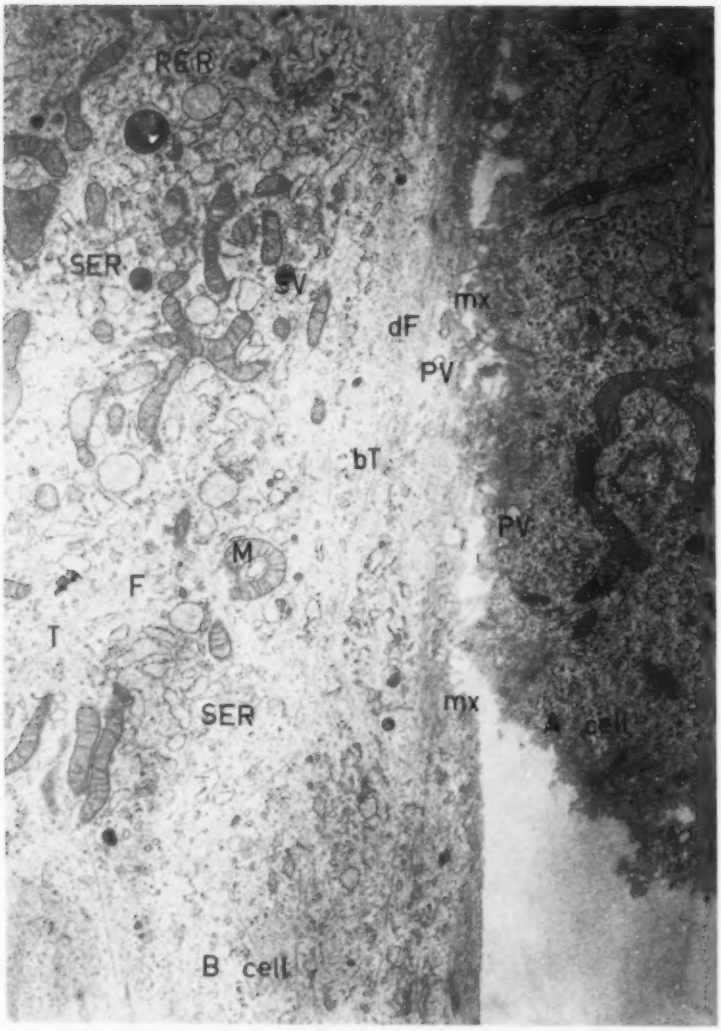

FIG. 5. Electron micrograph of type I HP cells from a young culture (lifespan completed : 0.5). The picture shows the interaction between two neighbouring cells (A and B). Note the high density of microfilaments which constitute a matrix (mx) sustaining the plasma membrane with discrete microfilaments (dF) and also bundles of microtubules (bT) inserted into this matrix. Both are parallel to each other. They are either more or less parallel to the plasma membrane (cell B) or perpendicular (cell A). In the cytoplasm, no preferential orientation appears for microtubules (T) and microfilaments (F). M : Mitochondria, RER : Rough endoplasmic reticulum, SER : Smooth endoplasmic reticulum, SV : Secretion vesicles, PV : Pinocytic vesicles, × 10,400.

microtubules appear perpendicular to the cell edge in one cell (A) and rather parallel in the second (B). Of course, because of possible oblique sections, the orientation of microfilaments and microtubules in relation to the plasma membrane is quite difficult to ascertain. In the type 2 cells (Fig. 6) we observed exactly the same features. However the cortical microfilament bundles seemed much more developed, they extended more deeply in the cytoplasm and their bundles looked less organized, probably in relation to the distorted outlines of these cells. As shown in Fig. 6, in some areas microfilaments have no peculiar orientation, in others they form large sheets, often with periodic densities, already described (Spooner *et al.*, 1971). Microtubules are very often in close relationship with the microfilament networks sustaining the plasma membrane. In two dimensional pictures the general orientation of microtubules compared to the cell shape is difficult to ascertain, and so at high magnification microtubules seem to have no specific direction. However, in limited areas, a unique direction is often observed. For example, in the upper

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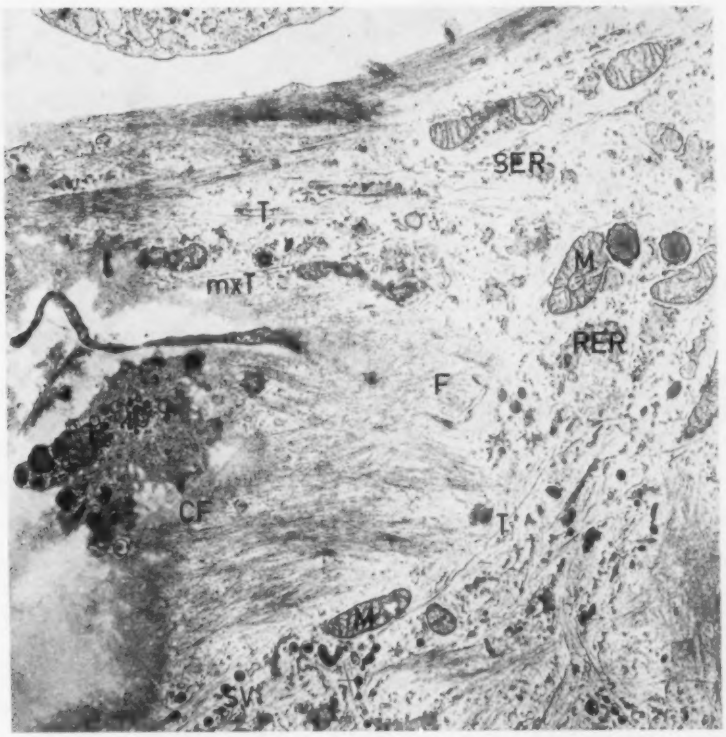

FIG. 6. Electron micrograph of type 2 HP cells from an old culture (life-span completed 0.95). The cortical microfilament bundles (CF) are much more abundant than in the type 1 cells with some of them running parallel to the plasma membrane and others extending deeply in the cytoplasm. Other microfilaments in the cytoplasm (F) do not have a particular orientation. Microtubules (T) are particularly abundant in this picture with some in close relationship to microfilaments of the matrix (mxT). M : mitochondria, RER : rough endoplasmic reticulum, SER : smooth endoplasmic reticulum, SV : secretion vesicles, × 10,400.

part of Fig. 6 microtubules are horizontally oriented and directed towards the cell edge located on the left side of the picture. The unique direction of microtubules is sometimes very striking as illustrated in Fig. 7 where all the subcellular organelles are parallely oriented to the cytoskeleton components. This specific orientation of subcellular organelles was particularly clear in type 2 cells since they contain abundant lipid droplets often disposed in rows and inserted in microtubule bundles. This observation was confirmed by red oil staining (not shown).

DISCUSSION

Having characterized a new strain of hamster fibroblasts (Raes and Remacle, 1983), we wanted to have more insight into the change of their cytoskeleton with *in vitro* ageing. Indeed, cytoskeleton components are ubiquitous in the cell and implicated in several cellular functions, so their possible impairment with ageing is quite an attractive hypothesis.

We observed a different pattern in the cytoplasmic microtubule complex (CMTC) in the type 2 cells, but as pointed out by de Mey *et al.*, 1978, cell shapes and degrees of spreading seem to predominantly determine the cytoskeleton pattern rather than the reverse. Two observations must be pointed out whatever their physiological significance might be:

1) The distribution of microtubules as well as microfilaments in the type 2 cells is a clear expression of the loss of polarity and of the homogeneous organization in these cells;
2) Some cytoplasmic areas seem completely devoid of microtubules and others of microfilaments. This is perhaps a way for these extremely large cells to face the problems of communication between the perinuclear and the peripheral regions, by creating some microtubule channels. The involvement of microtubules and

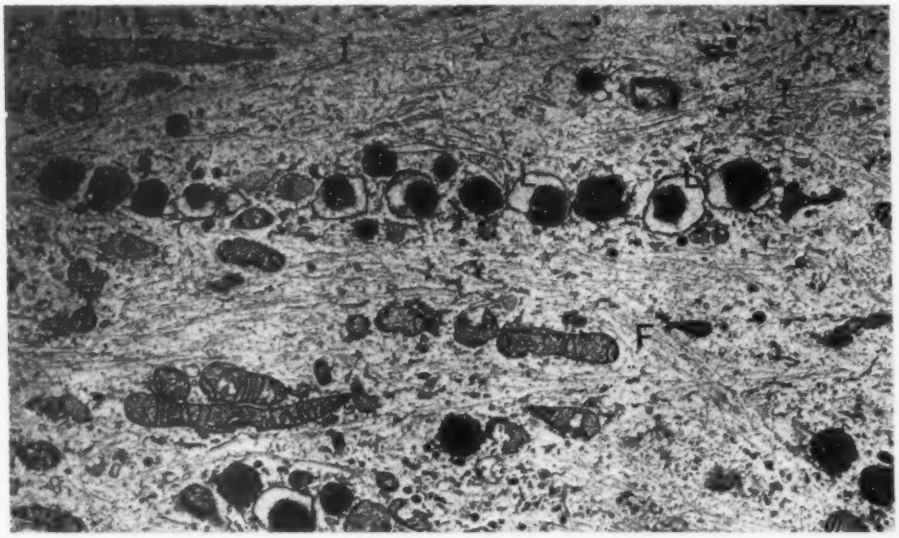

FIG. 7. Electron micrograph of type 2 HP cells showing the general orientation of the subcellular organelles given by arrays of microtubules and microfilaments. T. Microtubules, F. Microfilaments, L. Lipid droplets, × 21,200.

microfilaments in the movement of organelles has already been demonstrated (for a review, see Dustin, 1978).

The ultrastructural study of cytoskeleton is a difficult task because microfilaments and microtubules are labile structures difficult to preserve by the classical fixation and embedding procedures. That is why the immunocytochemical approach was used. However, TEM studies appeared useful, since they give some complementary information. The electron micrographs all show the dynamic state of cytoskeleton in the cortical area: when the predominant direction of bundles is parallel to the cell periphery it is clearly a stable cell edge; on the other hand, in moving cells in intercellular active contacts, the direction of the bundles is perpendicular to the cell edge (Vasiliev and Gelfand, 1977). The electron micrographs also confirm the extreme complexity of the cortical area in cultured cells (for a review, see Vasiliev and Gelfand, 1977; Franks and Wilson, 1977) and the interconnections between plasma membranes, microfilaments and microtubules in this area are far from being understood (Weatherbee, 1981). The apparently better preservation of microtubules and microfilaments in the cortical area is probably linked to their orientation in the plane of the section, since in this cytoplasmic area, the cytoplasm becomes much thinner. The comparative study of cytoskeleton by TEM with *in vitro* ageing, despite the limitations of these observations, confirms the immunocytochemical data at the ultrastructural level; this is particularly the case for microfilaments. If in some areas they appear organized in extremely large sheets, in others they appear clearly without organization and without orientation. The observations are in agreement with former ultrastructural studies of *in vitro* ageing mice embryo cells (Van Gansen *et al.*, 1979; Tuffery and Baker, 1973).

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To summarize, the cytoskeleton of HP differentiated type 2 cells is at least as developed as in the actively dividing type 1 cells but is characterized by a higher disorganization and a loss of polarity.

Microtubules are active structures which polymerize and depolymerize according to the cell needs. This active behaviour could be affected with ageing; one can imagine the alterations in the nucleation or polymerization process following for instance cytoplasmic alterations. Recently, the possible implication of calcium, magnesium ions (Staron and Jerzmanowski, 1981) and GTP (Kirschner, 1980) on the regulation of microtubules has been pointed out. Besides, unlowered levels of GSH seem necessary for microtubule stability and assembly: Oliver *et al.*, 1976 demonstrated that GSH oxidizing agents (as diamide) inhibit the microtubule assembly, normally induced by Con A binding on human leucocytes; Nath and Rebhun (1976) showed spindle dissociation by diamide in dividing sea urchin embryos. Modifications to the concentrations of these or other metabolites with *in vitro* ageing, seem quite conceivable. Moreover, changes in tubulin turn-over, in post-translational events such as tyrosylation (for a review, see Raff, 1979) or in the association of tubuline with microtubule-associated proteins (MAP) must also be taken into account. As a consequence of such alterations, the dynamic equilibrium of microtubules relative to the cell cycle could be drastically perturbated with severe implications for cell division and survival. If this is the case, these alterations could possibly be detected by treating cells with nocodazole, a very sensitive inhibitor of microtubule polymerization (Hoebeke *et al.*, 1976). Nocodazole probably favors depolymerization because when binding to free tubulin, it lowers the concentration of available free tubulin under the critical concentration. We couldn't provide evidence for a different susceptibility to the drug in the type 2 cells. However the interpretation, since type 1 and 2 cells

differ drastically in size and morphology, is all the more difficult. In the latter, however the depolymerization wave clearly starts from the nuclear region, whereas in the type 1 cells, it runs from the plasma membrane to the nucleus leaving a core of more stable microtubules at the focal point(s). With our present understanding of microtubules, we can propose some hypotheses for this differing behaviour. Recently, authors hypothesized the anchorage of microtubules to microtubule organizing centres (MTOCs) (Kirschner, 1980; Bergen and Borisy, 1980; De Brabander *et al.*, 1980). Although the exact nature and number of MTOCs is still controversial (for a review, see Solomon, 1980), in interphase cells (studied here), the centrosome seems to be the principal and most evident MTOC (Brinkley *et al.*, 1980 *a, b*).

The centrosome or centriolar complex is usually divided into 2 general component regions (for a review, see Peterson and Berns, 1980), the centrioles and a "cloud" of electron-dense, osmiophilic, surrounding material. Microtubules are abundant in this cloud material and Gould and Borisy, 1977 demonstrated that microtubules could be nucleated by this pericentriolar cloud, *in vivo* and *in vitro*. As pointed out by Peterson and Berns, 1980 the pericentriolar cloud could thus be the active microtubule organizing component. The anchorage of microtubules to the centrosome is still controversial. Microtubules are polar structures and there is some disagreement as to which end is anchored. According to Bergen *et al.*, 1980 and Kirschner, 1980, microtubules are probably anchored exclusively by their minus-end according to the polarity designation of Gould and Borisy, 1977. But McIntosh *et al.*, 1980 observed that microtubules grow with their minus-end free, at least from some MTOCs (kinetochores). De Brabander *et al.*, 1981 suggest a more open model where microtubules could be anchored at the minus or the plus end whilst other microtubules would not be anchored at all. In this way, cells dispose of microtubule sets of different polarities and stabilities. This open model could explain the behaviour of type 1 cells treated with nocodazole since the concentration of free tubulin, being decreased, microtubules depolymerize at their free ends, in this case from the plasma membrane to the MTOCs. Margolis and Wilson, 1981 proposed a lateral anchorage of tubules by dynein-like linkages, making treadmilling possible, with both ends free. Yet the behaviour of type 1 cells is not necessarily in disagreement with this model if MTOCs are surrounded by a local environment, favorable to tubulin assembly (De Brabander *et al.*, 1980) and thus protecting the microtubules from depolymerization even when not anchored. The behaviour of type 1 cells is also in agreement with the observations on tubulin repolymerization after tubule disruption. In each case, the repolymerization started mainly from the MTOCs and developed towards the cell periphery (De Brabander *et al.*, 1980; Watt and Harris, 1980).

The problem is how to explain the centrifugal depolymerization wave of the differentiated type 2 cells. Is their anchorage looser? Is the polarity of the tubules reversed? Is the protecting environment of the MTOCs less efficient? A fine ultrastructural study of the centriole area respectively in type 1 and 2 cells would certainly be a worthwhile prospect. Or are the microtubules in these type 2 cells anchored more firmly to the plasma membrane? There is some evidence for the interaction of microtubules and tubulin with membranes, and more particularly with the plasma membrane (for a review, see Weatherbee, 1981). A bridge molecule has been hypothesized, probably a dynein-like protein, that binds the cytoplasmic tubulin to a membrane bound tubulin. This linkage could be stabilized better in an old environment compared to the MTOC anchorage.

Whatever the exact mechanism beyond the above observations, it will have to explain the differing pattern of tubule depolymerization in the same cells, but at different stages of their differentiation and history. In this way, HP ageing cells represent a unique system in order to study the different factors inherent to microtubule composition or present in the cytoplasm (GTP, GSH, pH, . . .) and which condition the polarity of the microtubules.

Considering the physiological implications of these findings, we would like to know if the change in depolymerization polarity observed in type 2 cells could impair cell division. If this is the case, this modification could explain the sudden drop in cell division associated with this differentiation process and play a determinant role in cell ageing.

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THE SKIN TEMPERATURE GRADIENT IN THE UPPER LIMBS OF OLD WOMEN

TREVOR H. HOWELL

Professorial Department of Geriatric Medicine, St George's Hospital, London, SW17, England.

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Abstract—In the upper limbs of 103 convalescent old women, there was a fall of skin temperature between axilla and thumbs equal to 4.8°C. Variations in temperature became wider from proximal to distal sites, being 3.2° in the right axilla and 3.4°C in the left; compared with 14.0° in the right thumb and 13.6°C in the left. Skin temperature could be correlated with circumambient air, but not with age.

INTRODUCTION

COLD HANDS are not uncommon among the elderly. A previous investigation into skin temperature gradients of the lower limbs (Howell, 1982) showed that there was a mean fall of 8.8°C between the groins and the toes in a series of aged women. The present paper, using the same subjects, demonstrates the falling temperature gradient in the upper limbs, from axilla to thumb.

MATERIAL AND METHOD

The subjects examined comprised 103 convalescent elderly women under the care of the writer at Queen's Hospital, Croydon. Their ages, as shown in Table 1, ranged from 61 to 100 years. The skin temperatures were taken with an electric thermometer, made by Light of Brighton, which had been tested to an accuracy of ± 0.2°C. Readings were taken deep in both axillae, the antecubital fossae, palmar surfaces of both wrists and the pulp of both thumbs. All measurements were checked by two observers. Room temperature was noted at the time of recording. Examination always took place between 9:30 and 11:00 A.M. The whole research took just under a year to complete.

RESULTS

Skin temperatures in the right axilla varied from 34.2° to 37.4°C. This gave a range of 3.2° with a mean temperature of 35.8°C. There was a standard deviation of 0.68°. In the left axilla, the range was from 34.0° to 37.4°C, the difference being 3.4°C. This gave a mean of 35.7°C, while the standard deviation was 0.61°. As noted in a previous paper (Howell, 1972) there might be considerable differences between the right and left axillae, sometimes as great as 1.4°C. Hence, the concept of a single "normal" axillary temperature is therefore unsatisfactory.

The temperature for the elbow was taken in the mid-line of the antecubital fossa. The

TABLE 1. AGE OF SUBJECTS

Age Group	*n*
61–70	11
71–80	39
81–90	41
91–100	12
Total	103

minimum reading on the right side was 27.6° and the maximum was 36.8°C. This gave a range of 9.2°. The mean temperature was 34.6°C with a standard deviation of 1.32°. On the left side, the minimum was 27.4° and the maximum 37.0°C. The variation was 9.6°. The mean figure was 34.6°C, with a standard deviation of 1.19°.

Readings for the wrists were taken in the mid-line of the palmar surface. On the right, the minimum reading was 23.4° and the maximum was 36.2°C. This gave a range of 12.8°. The mean temperature was 32.6°C and the standard deviation 2.24°. On the left, the lowest reading was 26.0° and the highest 36.8°C. The range was 10.8°. There was a mean of 32.7°C and a standard deviation of 2.24°.

When the temperature at the centre of the pulp of the thumb was read on the machine, the minimum on the right side was 22.2° and the maximum was 36.2°C. Hence, the variation amounted to some 14.0°. The mean of the readings came to 30.7°C with a standard deviation of 3.62°. On the left side, the minimum was 22.8° and the maximum 36.4°C. This gave a range of 13.6°. The mean came to 31.3°C and the standard deviation 3.43°.

It will be noted that the minimum and the maximum readings tended to diminish peripherally from axilla to thumb. Hence, the mean figures did the same. Similarly, standard deviation tended to become greater as the distal part of the limbs were approached. This is shown clearly in Table 2.

STATISTICAL CONSIDERATIONS

It is possible to say that there was a definite gradient of fall in temperature from axilla to thumb pulp, as shown in Table 2; the average drop being 4.8°C, taking the mean readings of right and left limbs. This steady fall of temperature was accompanied by in-

TABLE 2. TEMPERATURES AT SELECTED SITES (°C)

Site	Minimum	Maximum	Range	Mean	Standard Deviation
Right axilla	34.2	37.4	3.2	35.8	0.68
Left axilla	34.0	37.4	3.4	35.7	0.61
Right elbow	27.6	36.8	9.2	34.6	1.32
Left elbow	27.4	37.0	9.6	34.6	1.19
Right wrist	23.4	36.2	12.8	32.6	2.24
Left wrist	26.0	36.8	10.8	32.7	2.18
Right thumb	22.2	36.2	14.0	30.7	3.62
Left thumb	22.8	36.4	13.6	31.3	3.43

TABLE 3. CORRELATION COEFFICIENTS, ROOM TEMPERATURE

Site	Right side correlation	Left side correlation	Significance right	Significance left
Axilla	T = 3.57	T = 2.828	highly	significant
Elbow	T = 2.376	T = 1.749	probably	not significant
Wrist	T = 4.655	T = 3.680	highly	highly
Thumb	T = 3.746	T = 3.688	highly	highly

creased range of readings. The variations in thumb pulp were 14.0° on the right and 13.6°C on the left; this compared with 3.2° in the right axilla and 3.4°C in the left.

The standard deviation of the temperature readings also increased from axilla to thumb. Figures for the axillae were 0.68° on the right and 0.81° on the left. The right elbow showed 1.32° for the right and 1.16° for the left. As regards the wrists, the deviation was 2.24° on the right and 2.18° on the left. The right thumb readings gave 3.62° for the right and 3.43° for the left. This may be seen in the last column of Table 2.

When correlation of readings with room temperature was studied, there seemed to be either a significant or highly significant finding in all sites except the left elbow. At the right axilla, T = 3.57 which is highly significant. At the left axilla, T = 2.828 which is significant. The right elbow had a correlation coefficient of T = 2.376 which is probably significant. The left elbow, with T = 1.749 seems not to be significant, however. For the right wrist, T = 4.655 and for the left, T = 3.680, both are highly significant. The right thumb showed T = 3.746 and the left had T = 3.688, both highly significant. Such figures suggest the skin temperature readings in the upper limbs of aged women can be correlated significantly with room temperature in the circumambient air. (Table 3)

On the other hand, there was an absence of correlation between the age of the subjects and the skin temperature readings, as may be seen in Table 4. No site showed any significance. The axillary figures for correlation were T = 0.271 and T = 0.766, respectively. At the elbow, T = 0.109 and 0.176. For the wrist, T = 0.74 and 0.534 on the right and left sides. At the thumbs, T = −0.6 on the right, while T = 0.14 on the left. These figures may be seen in Table 4.

The differences between right- and left-sided readings in axillae, antecubital fossae wrists and thumbs are shown in Figs. 1, 2, 3, 4 and 5. It would seem that there are no really significant differences between any of the sites mentioned.

DISCUSSION

The temperature gradient in the upper limb of old women is much less marked as compared with the lower limb. While the mean fall from groin to toe amounted to 8.8°C

TABLE 4. CORRELATION COEFFICIENTS WITH AGE

Site	Correlation right side	Significance	Correlation left side	Significance
Axilla	T = −0.6	Nil	T = 0.14	Nil
Elbow	T = 0.74	Nil	T = 0.584	Nil
Wrist	T = 0.109	Nil	T = 0.176	Nil
Thumb	T = 0.271	Nil	T = 0.766	Nil

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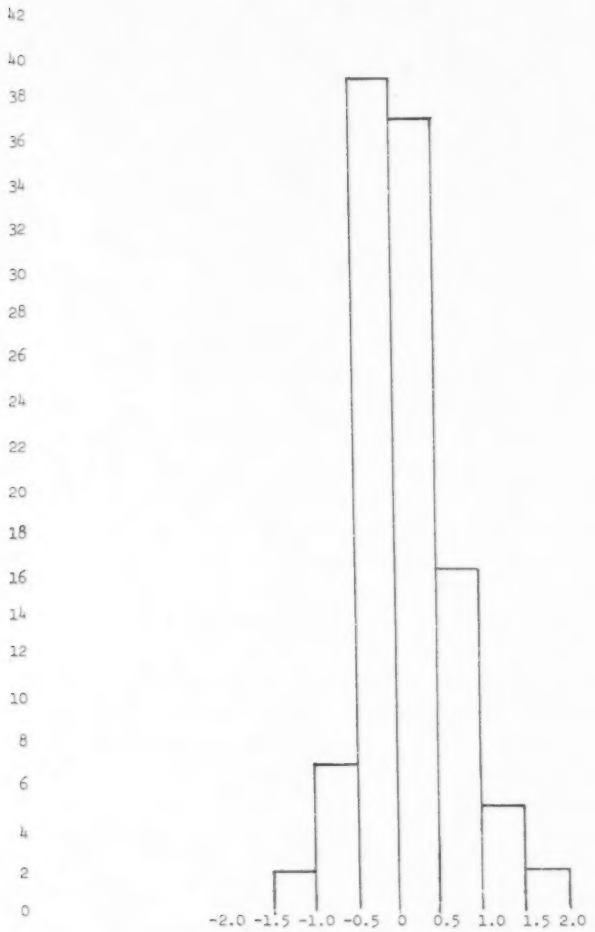

FIG. 1. Temperature difference in Axillas (right-left side) 103 females aged 61–100.

(Howell, 1982), the fall in the upper limbs of the same group of subjects only came to 4.8°C, as from axilla to thumb. There seemed to be relatively little drop in maximum readings at the various sites. As may be seen from Table 2, both axillae had maximal readings of 37.4°C, while thumbs showed 36.2° on the right and 36.4°C on the left. In fact, the right elbow and the left wrist had figures which were the same, i.e., 36.8°C. As regards minimum temperatures, the fall is much greater, from 34.2° in the right axilla to 22.2°C in the right thumb; and from 34.0° in the left axilla to 22.8°C in the left thumb. This difference between maximum and minimum figures is not dissimilar to the findings in the lower limbs, noted in the previous paper, (Howell, 1982). The findings suggest that the number of old women whose temperature falls in the upper limb is quite small; while there are others whose thumbs are much colder than their axillae. It is, presumably, this latter group who have an arterial supply which is not really adequate for their peripheral

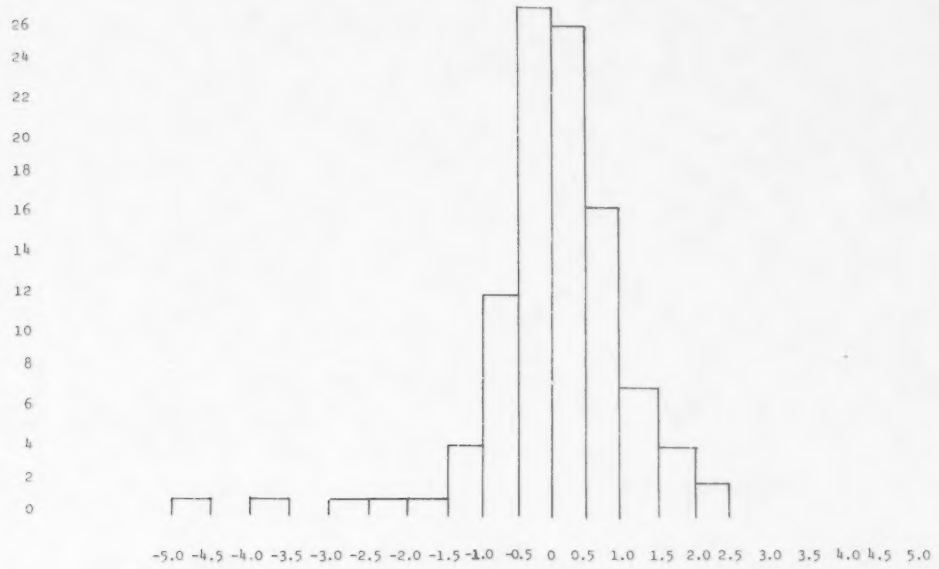

FIG. 2. Temperature difference in elbows (right-left side) 103 females aged 61–100.

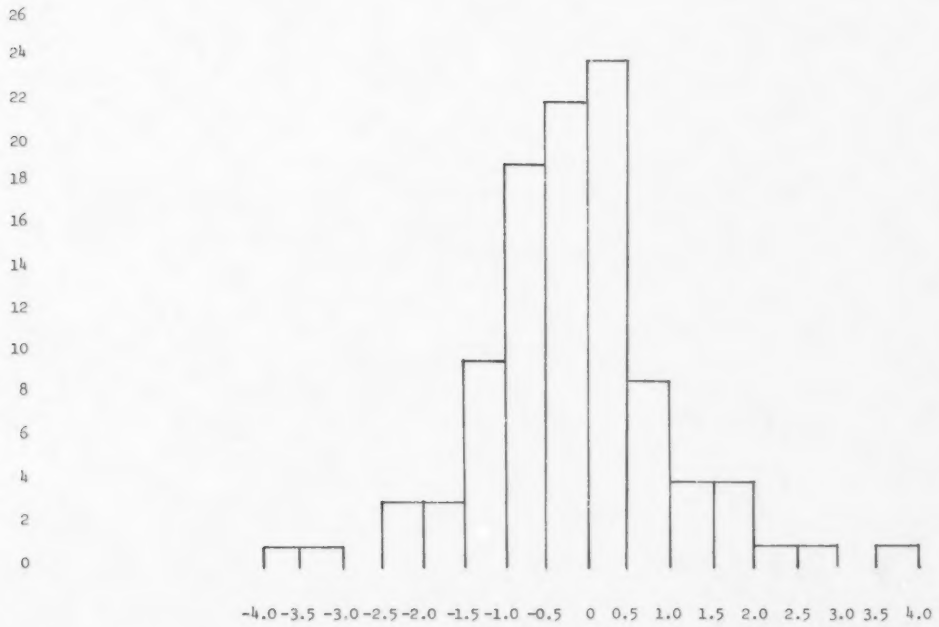

FIG. 3. Temperature difference in wrists (right-left side) 103 females aged 61–100.

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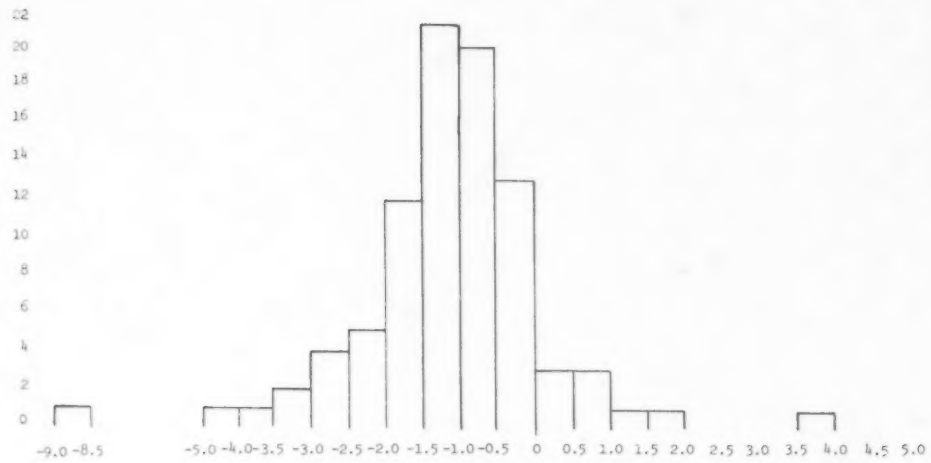

FIG. 4. Temperature difference in fingers (right-left side) 103 females aged 61–100.

tissues, especially in cold weather, since the correlation of skin temperature with room temperature is usually so highly significant.

CONCLUSIONS

In a group of 103 convalescent old women, there was a fall of skin temperature between axillae and thumbs amounting to 4.8°C. The variations in temperature become greater from proximal to distal sites of readings. Ranges of 3.2° and 3.4°C were seen in right and left axillae. The variation at the thumbs was 14.0°C on the right and 13.6°C on the left. Skin temperature readings could be correlated with the temperature of the circumambient air but not with age.

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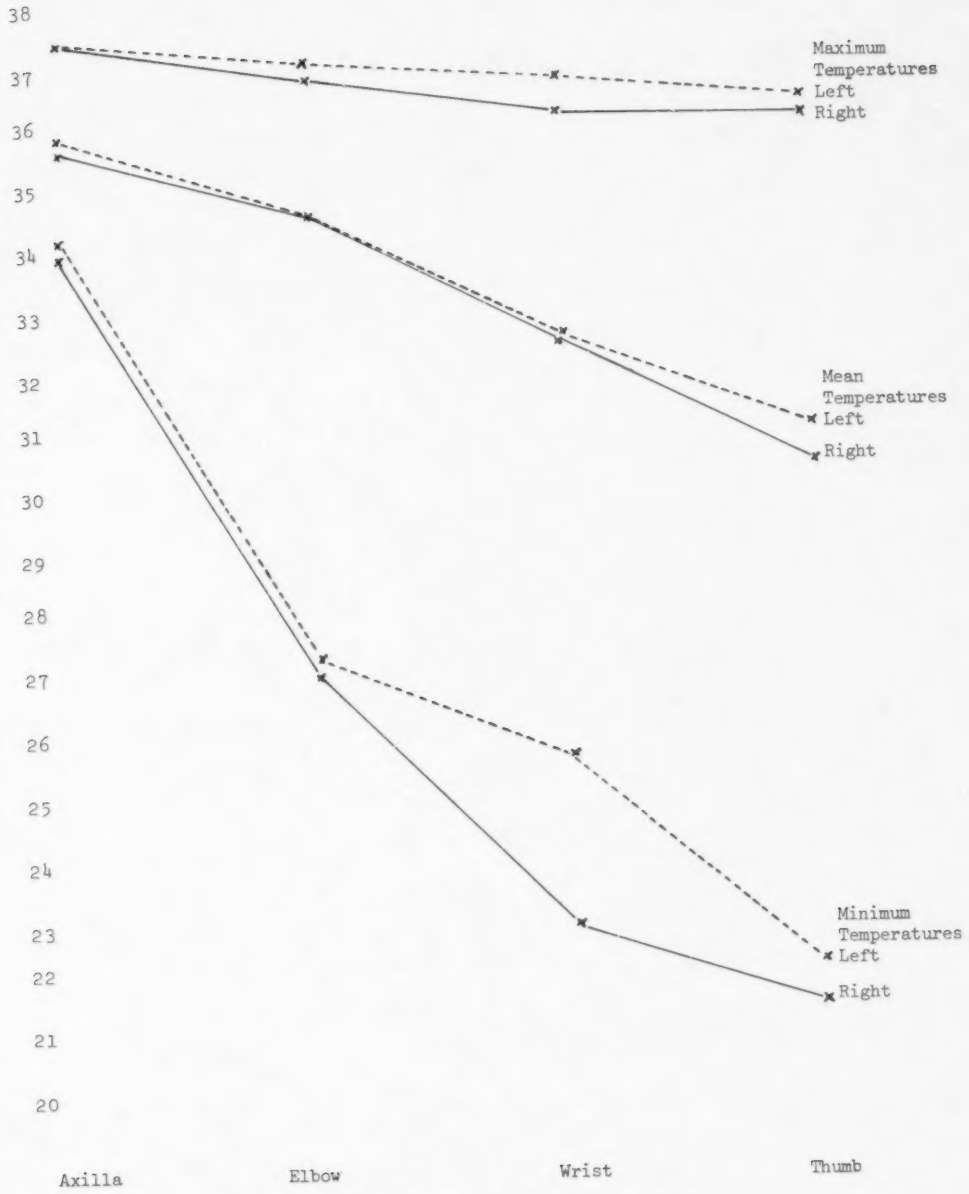

FIG. 5. Temperature gradient in upper limbs.

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AGE-SYNCHRONOUS CULTURE OF *CAENORHABDITIS ELEGANS*: TECHNIQUE AND APPLICATIONS

P.M. FINDEIS*, C.J. BARINAGA, J.D. WILLETT, and S.O. FARWELL

Department of Chemistry, University of Idaho, Moscow, Idaho 83843

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Abstract—The inclusion of mobility to an age synchrony method and the development of an inexpensive filter device resulted in a natural model aging system without resorting to invasive chemical techniques. Large or small cohorts of nematodes with less than 1% offspring contamination are possible. The filter/mobility method is compared to other methods using the same strain and culture temperature. The applicability of the method is shown with a variety of parameters, and a previously reported parameter of aging in nematodes, *i.e.*, specific gravity, is shown not to be an aging parameter of *Caenorhabditis elegans*.

INTRODUCTION

THE USE of free-living nematodes, particularly *Caenorhabditis elegans*, in aging and development studies has been advocated by a number of workers (Gershon, 1970; Zuckerman *et al.*, 1971; Edgar & Wood, 1977; Gandhi *et al.*, 1980; Bolanowski *et al.*, 1981; Hosono *et al.*, 1982). Unfortunately, there has been an inability to obtain synchronously-aging adults in large numbers that are not damaged, chemically or genetically altered, or contaminated by offspring generations.

Various partially successful methods have been attempted. One method was to allow gravid adults to deposit eggs on agar for a definite time, *e.g.*, 4 hours, and subsequently remove or kill the adults (Byerly *et al.*, 1976). This created an "egg window" with the eggs being the start of a synchronous population. Egg-window synchrony broke down when the nematodes matured and began to produce offspring of their own (Mitchell *et al.*, 1979). To maintain age synchrony, separation of the cohort from its progeny was needed. In small populations of less than 100, this was done by transferring the individual adults with a stylus (Hosono, 1980; Bolanowski *et al.*, 1981) or pipette (Mitchell *et al.*, 1979) every 2 days. For obvious logistical reasons, this method is of limited use in generating biochemically-significant quantities of senescent, free-living nematodes.

A second method was to inhibit reproduction of the progeny with either DNA synthesis inhibitors (Kisiel *et al.*, 1972; Mitchell *et al.*, 1979; Hosono, 1978b; Gandhi *et al.*, 1980) or by elevated culture temperatures (Hieb & Rothstein, 1975). Unfortunately with this approach, non-specific damage occurred to the nematode (Zuckerman, 1976) and inhibition

*Author to whom correspondence should be addressed.

of spermatogensis was possible only in those strains having a temperature sensitive mutation.

A third method was to separate the fertile adults from their young by physical means on a daily basis, be it filtration (Tilby & Moses, 1975), differential settling (Klass, 1977), or both (Bollinger & Willett, 1980). Unfortunately, the phenomenon of individual adults containing hatched progeny, *endotokia matricida* or "uterine hatch," caused synchrony to breakdown and become increasingly incomplete by the inclusion of subsequent generations into the synchrony population.

This paper describes a method that uses an inexpensive, semi-micro filtration device, and utilizes nematode mobility to eliminate uterine hatch and to obtain either small or mass cultures of synchronously-aging adults. The significance of length, weight, specific gravity, total protein, and osmotic fragility as aging parameters and the applicability of specific gravity to a synchrony scheme are also examined. Further, the inapplicability of phthalate esters for specific gravity determination and of specific gravity as an aging parameter are demonstrated.

MATERIAL AND METHODS

Caenorhabditis elegans var. Bristol, strain N2, was stock cultured on lawns of *Escherichia coli* on NG agar (Brenner, 1974) at 20 °C as described by Bollinger & Willett (1980).

Filtrations were performed with commercially available polyethylene scintillation vials (VP-1; Isolab Inc., Akron, OH) modified by cutting off the bottom of the vials and cutting a 20 mm hole in the cap. Squares of Nitex (Tetko Inc., Elmsford, NY) (4 cm × 4 cm) were placed between the caps and vials and screwed on to yield autoclavable filters costing less than $0.15 each in materials. Smaller filtration devices, constructed in a similar manner from 7 mL MiniVials (Kimble, Inc.) and squares of Nitex (2.2 cm × 2.2 cm) were tested and found to be more inconvenient. Nitex is a high-strength, woven nylon cloth; its suffix number is the nominal mesh opening (width between threads) expressed in micrometers.

Synchrony

Synchrony populations were established in the following manner with all washes and rinses using M9 buffer (Brenner, 1974). A 4 day-old stock culture was filtered through Nitex 10 and a minimum of 1,000 filtrate juveniles was transferred to an *E. coli*/NGS lawn (Bollinger & Willett, 1980), forming the first pre-synchrony population. After three days, this population was washed and filtered through Nitex 10. Juveniles in the filtrate were transferred to an *E. coli*/NGS lawn forming the second pre-synchrony population. After 2.5 days, the second pre-synchrony population was washed onto Nitex 30 and rinsed 10 times. Nematodes on the filter were transferred to fresh *E. coli*/NGS lawns. After 24 hours, this population (then including eggs and first stage juveniles) was filtered through Nitex 10. The filtrate juveniles (hatched only within the preceding 12 hours) formed the start of a synchrony population and were transferred to *E. coli*/NGS lawns with a population density no greater than 5×10^4 juveniles per lawn. Two days later, the nematodes were washed onto Nitex 30 filters, rinsed, and transferred to *E. coli*/NGS lawns. At 3.5 days after hatch, and on a daily basis thereafter, adult populations were re-synchronized by filtration onto Nitex 41, washing six to ten times, and re-culturing on *E. coli*/NGS lawns. After 8.5 days, *E. coli* lawns were grown on NGF agar (identical to NGS agar containing no dextrose) to prevent lawn overgrowth in the presence of post-reproductive adults.

During the period when uterine hatch occurs, 4.5 to 8 days after hatch, nematode mobility was used to select against adults exhibiting uterine hatch. After washing on Nitex 41, the adults were back washed off the filter into a 15 mL conical centrifuge tube and allowed to settle completely to less than 0.75 mL. The suspension of adults was dropped onto a small central area of the lawn and that area outlined with marker. In a short time, the nematodes have moved out of the central area to feed into the lawn, leaving behind the dead or infirm resulting from uterine hatch. Approximately five hours later, the drop zone was microscopically inspected and then excised if a majority of individuals were dead or suffered from uterine hatch.

Survival Curves

Survival curves were determined on populations at densities of 1 worm/cm^2 and 265 worms cm^2. Low-density cohorts, fewer than 200 individuals, were cultured on lawns 10 cm in diameter and high-density cohorts, more than 10,000 individuals, were cultured on lawns 15 cm in diameter. All wash solutions were refiltered on Nitex 20

to determine any losses of the original cohort through the Nitex 41 filters. All filtration devices were microscopically examined for any untransferred nematodes.

Physical Dimensions

Length and width were determined optically on nematodes anesthetized with either 0°C M9 or 0.5% phenoxypropanol in M9.

Dry weight per individual worm as a function of age was determined on a minimum of six replicate samples of large populations at each specified day. Nematodes sampled were heat killed at 60°C for 5 minutes, washed with chilled 18 megaohm distilled-deionized water, and chilled in ice water. The worms in a 10 μL aliquot (SMI Micropettor B) were replicate counted under a dissecting microscope before and after transfer to tared aluminum boats (12 mm diameter). These samples were dried to constant weight in a vacuum dessicator over P_2O_5. Weighing was done on a Cahn G-2 electrobalance, and sample size varied from 14 to 524 individuals. Dry weight controls consisted of 10 μL aliquots of chilled water solution containing no nematodes.

Protein

Protein was determined by a modification of the Lowry technique according to Peterson (1977).

Osmotic Stress

Osmotic fragility was determined with modification to the method of Bollinger & Willett (1980) by exposing nematodes to M9 buffer diluted 10,000 times with 18 megaohm water and observing the individuals after 30 minutes. Nematodes were scored survived if they were swimming or moving, as compared to stunned and non-moving, or burst, *i.e.*, intestinal contents ruptured through the vulva.

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Specific Gravity

Specific gravity was determined by discontinuous gradient centrifugation using isotonic mixtures of M9 buffer and 548 mM metrizamide (Sigma Chemical Co., St. Louis, MO). Approximate stock metrizamide concentration was determined from Loos & Roos (1976). Solution specific gravities were experimentally determined by six replicate comparisons of fixed volume to 18 megaohm water at 20°C; specific gravities ranged from 1.059 to 1.154 gm/cm³ in increments of 0.014 gm/cm³. At daily transfer, aliquots of 50 to 1,000 individuals from four replicate populations were added to the top of a stepwise gradient. Each gradient consisted of a minimum of 5 layers, each layer a minimum of 1 cm in depth (approx. 180 μL) in semi-micro centrifugation tubes constructed from 10 cm lengths of Pyrex tubing (6 mm OD, 4 mm ID) flame sealed at one end. Nematodes were centrifuged at 15,000 × g in a swinging bucket rotor for 10 minutes at 20°C. Tubes were chilled in ice water and the number of individuals at each interface and in each band was counted. Maximum specific gravity distribution was calculated by assigning the specific gravity of a band to a nematode if the nematode was in the band or at the upper interface of the band. Specific gravity determinations in organic non-isotonic media (Zuckerman *et al.*, 1972; Kisiel *et al.*, 1974) were attempted.

A theoretical specific gravity-age function was calculated from the data of Kisiel *et al.*, (1974) in an attempt to compare previous data obtained on *C. briggsae* to *C. elegans*. *C. briggsae's* age at the start of reproduction, age at the end of reproduction, and generation time (4.5 days, 14 days, and 5.3 days respectively) were compared to *C. elegans'* age at the start of reproduction, age at the end of reproduction, and generation time (3 days, 7 days, and 3.5 days respectively). The age of *C. briggsae* was adjusted to match *C. elegans'* age at these biological milestones, but the specific gravity was kept unchanged between *C. briggsae's* original and adjusted age. A daily theoretical specific gravity range could not be calculated as no standard deviation was given in the original publications.

Statistics

Statistics of the mean specific gravity at each age of replicate cohorts were performed using preprogrammed SAS (Statistical Analysis System, SAS Institute, Raleigh, NC) for analysis of variance and Duncan's multiple range test, or according to Steel & Torrie (1980) for Tukey's *w* procedure.

RESULTS

Figure 1 shows body length and fecundity in replicate high-density populations (> 10,000 worms) as a function of age. Lengths shown are typical of *C. elegans*, N2, cultured at 20°C in the filter/mobility synchrony system. Fecundity determinations

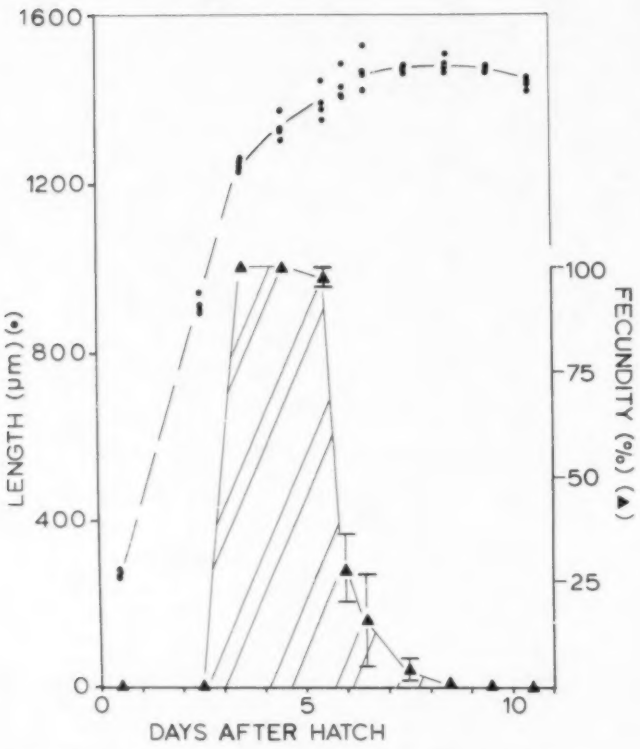

FIG. 1. Fecundity and total body length in four replicate high-density populations of *C. elegans*, N2. Values (•) represent mean total body length of 20 worms sampled from each cohort; standard deviation bars are omitted for the sake of clarity. Values (▲) represent the percentage of the cohorts that are fecund (i.e., worms that hold at least one egg that has developed into a distinctly visible embryo) ± standard deviation; standard deviation bars are omitted when they would be encompassed by symbol. Striped region indicates the reproductive phase of the life cycle.

beyond 6 days after hatch are hindered due to the retention of unfertilized, highly granular eggs. No attempt was made to quantify the number of eggs per individual due to the large number contained within the individual during the peak reproductive period, 4 to 6 days after hatch.

Figure 2 shows the survival curves of replicate low density (< 200 worms) populations. Beyond seven days after hatch positions of worms were noted after and prior to transfer. A worm not moving for 24 hours was scored as dead; this scoring was confirmed by dissolution of the intestinal structure during the next two days. Worms developing uterine hatch were not excluded from the survival curve.

Tables 1 and 2 show that population density has no effect on growth and development in this system. These data also compare worms cultured with the filter/mobility method vis-à-vis other monoxenic culture methods. No comparison is made to axenic or liquid culture methods due to the variables of caloric restriction, continuous movement, and sensitivity of *C. elegans* to bacterial concentration in liquid culture.

Table 3 shows the results of other possible parameters of aging in *C. elegans* and com-

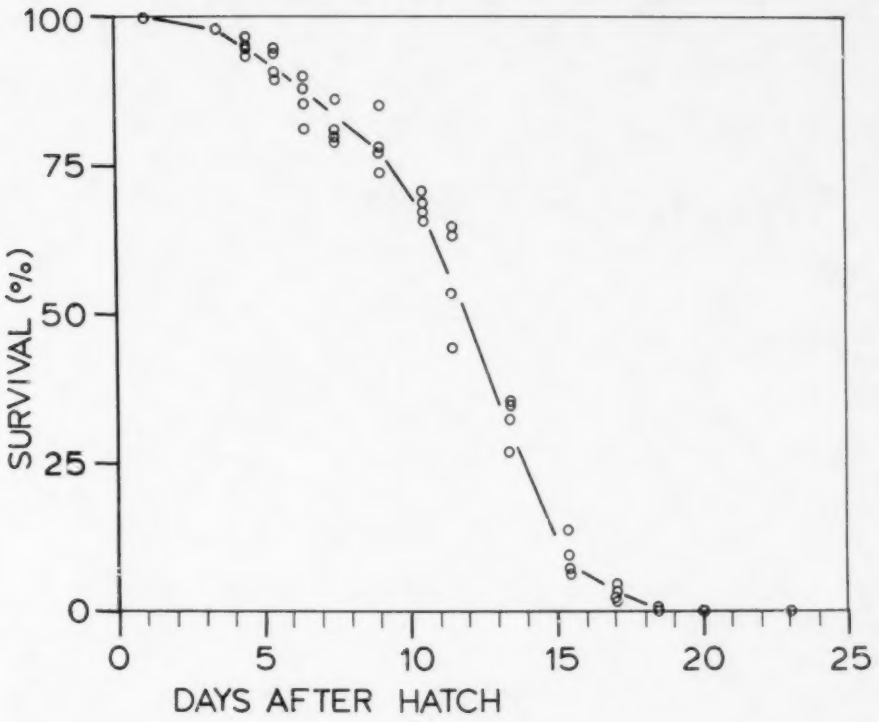

FIG. 2. Composite survival curve of four replicate, low-density cohorts of *C. elegans*, N2. Values represent percent living of four populations (initial n = 104, 88, 95, 70).

TABLE 1. COMPARISON OF POPULATION DENSITY AND SUBSEQUENT GROWTH

Population density (worms/cm²)	*Length ± SD (μm)*	*Individuals sampled*
1.0	1002 ± 204	4
2.5	1188 ± 116	5
9.0	1100 ± 120	18
32	1187 ± 142	62
36	1075 ± 196	44
265	981 ± 145	51

Nitex 10 filtrate juveniles from the adults of a second pre-synchrony population were transferred to *E. coli*/NGS lawns, 16 mm diameter (one well of a Costar 24, Costar Inc., Cambridge, MA), individuals counted, and cultured at 20°C. Counts were confirmed during subsequent days. After 62 hours, the worms were anesthetized and measured optically. A minimum of 44 individuals of the population was sampled; if the population numbered under 20, the entire population was measured.

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TABLE 2. COMPARISONS OF POPULATION AND SYNCHRONY PARAMETERS OF *C. ELEGANS*, N_2, MONOXENICALLY CULTURED, 20°C

Parameter	*Filter/Mobility Method*	*Literature Values (Method)*		*Reference*
Total Length at 3.5 days after hatch (μm)	1250 ± 50	1470 ± ?	(a)	Hosono, 1978b
		1410 ± ?	(b)	Hosono, 1978b
		1280 – 1350	(c)	Hosono, 1978a
		1260 ± ?	(d)	Mitchell *et al.*, 1979
		1210 – 1235	(i)	Byerly *et al.*, 1976
		1130 – 1230	(e)	Hirsch *et al.*, 1976
Median Survival (days)	11.9 ± 0.51	9.8 – 16	(e)	Hoson *et al.*, 1980
		14.5 ± 2	(e)	Hirsh *et al.*, 1976
		14.5 ± 2	(c)	Klass, 1977
		11.4 – 12.2	(f)	Bolanowski *et al.*, 1981
		12.5 – 12.8	(e)	Hosono *et al.*, 1982
Length of reproductive phase (days)	4 – 5	2.71	(g)	Byerly *et al.*, 1976
		3	(a)	Hosono, 1978b
		3 – 5	(h)	Bollinger & Willett, 1980
		6	(c)	Klass, 1977
Start of egg-laying (hours from hatch)	56 ± 5	48	(c)	Klass, 1977
		45	(a)	Hosono, 1978b
		55	(i)	Byerly *et al.*, 1976
		60 (?)	(h)	Bollinger & Willett, 1980
Maximum Survival (days)	18.3 – 22	15.5 – 23	(e)	Hosono *et al.*, 1980
		20	(c)	Klass, 1977
		17 – 20	(f)	Bolanowski *et al.*, 1981

Methods used: (a) egg window; (b) egg window with subsequent DNA-synthesis inhibitor; (c) hatch window; (d) hatch window, Pt wire transfer; (e) hatch window, toothpick transfer; (f) egg window, toothpick transfer; (g) egg window, needle transfer @ 19.5 C; (h) filter/settling; (i) egg window @ 19.5 C.

pares the survival percentage of typical populations cultured via filter/mobility with reported values using the filter/settling method. Contamination of the experimental cohort by uterine hatch using filter/mobility method was less than 0.5%. Typically 1% of the live population is lost with use of drop-zone excission in this synchrony method. High incidence of uterine hatch could be induced if the population exhausted its food supply for any significant time during the peak reproductive days. Use of only three drop-zone excissions during a 36 hour period in the culture of such populations decreased uterine hatch from 35% at six days after hatch to less than 1% at 7.5 days after hatch.

Figure 3 depicts a possible biological aging parameter as a function of age in *C. elegans*. Osmotic fragility is a physiological stress test and, as expected, the organism exhibited declining ability to withstand stress as it aged. The decline in the ability to withstand stress is another indication that the cohorts were indeed aging.

An investigation of specific gravity as an aging parameter is shown in Figures 4 and 5 and Table 4. Mean specific gravities in isotonic metrizamide/M9 buffer solutions of four replicate cohorts before hatch and at various ages after hatch is shown in Figure 4.

TABLE 3. PHYSICAL PARAMETERS AND SURVIVAL IN HIGH-DENSITY COHORTS OF *C. ELEGANS*.

Time after hatch (days)	*Filter/mobility survival (%)*[A]	*Reported survival (%)*[B]	*Individual dry weight (ng)*	*Individual Protein*[C]	*Total Body length (μm)*	*Body width (μm)*
0.5	100 ± 6.8		8 ± 3	270 ± 17.4	12.6 ± 1.0	
1.5			21 ± 9			
2.5	98.8 ± 9.2	100	310 ± 22	206 ± 64	918 ± 77.0	49.1 ± 4.0
3.5		84	778 ± 34	441 ± 34	1248 ± 47.4	64.7 ± 2.8
4.5	97.1 ± 23.7		938 ± 45	698 ± 122	1342 ± 63.7	69.1 ± 3.1
5.5		80	892 ± 65	631 ± 82	1395 ± 46.9	70.6 ± 2.9
6.5	80.5 ± 5.6			522 ± 43	1470 ± 60.5	73.0 ± 3.5
7.5		47	813 ± 35	590 ± 55	1474 ± 58.4	74.8 ± 4.4
8.5	63.3 ± 2.4			573 ± 103	1482 ± 70.3	76.3 ± 4.1
9.5	63.3 ± 16.6	34	720 ± 45	465 ± 54	1470 ± 74.2	77.5 ± 4.8
10.5	53.8 ± 9.9			534 ± 96	1439 ± 64.4	77.2 ± 5.3
11.5		19	684 ± 42	561 ± 76		
12.7	42.6 ± 6.8					
13.5		7				
14.1	25.9 ± 7.1					
16.7	7.0 ± 3.5					

Note—Values express mean ± 1 standard deviation.

A—High-density (25,000 worms) population survival was determined by count of quadruplicate aliquots (0.25% @) of live worms at specified days. The large uncertainty was due to the inability to keep worms in uniform suspension for sampling.

B—Data taken from Bollinger & Willett (1980), no standard deviation was given.

C—Value ± 1 standard deviation expressed in nanogram equivalents of bovine serum albumin.

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Analysis of variance indicated that the mean specific gravity values of all age groups are not the same ($P < 0.0001$) and that age accounted for 92% of the variation in specific gravity. Duncan's multiple range test indicated that specific gravity did not vary in a continuous manner as a function of age, as reported by previous workers in a closely-related free-living nematode (Kisiel *et al.*, 1974; Zuckerman, 1976). Significant differences at the 5% or 1% confidence level according to the Duncan's multiple range test (Table 4), and confirmed by Tukey's *w* procedure, indicated that specific gravity varied in a manner coincidental with the gravidity and the fecundity (the fertility or viability of the eggs) of the adult.

Isotonic metrizamide/M9 solutions appeared to be innocuous to *C. elegans*. Gradient centrifugation tubes had to be chilled in ice water to stop movements of the nematodes that would disrupt the interface between bands. Worms swam vigorously after exposure of 90 minutes to metrizamide at room temperature. A different effect occurred with exposure of *C. elegans* to phthalate esters, Figures 5a–f. Centrifugation of nematodes in metrizamide longer than 10 minutes at 15,000 × g failed to change the final position of any nematode. Centrifugation of nematodes in capillary tubes of phthalate esters (Zuckerman *et al.*, 1972) forced the nematodes to adhere only to the glass surfaces of the centrifugation tube and, as shown in Figure 5, to slowly dissolve with exposure to dimethyl phthalate-dibutyl phthalate solutions. The same dissolution phenomenon is possible by toothpick transfer of an adult off agar and immersion in phthalate esters.

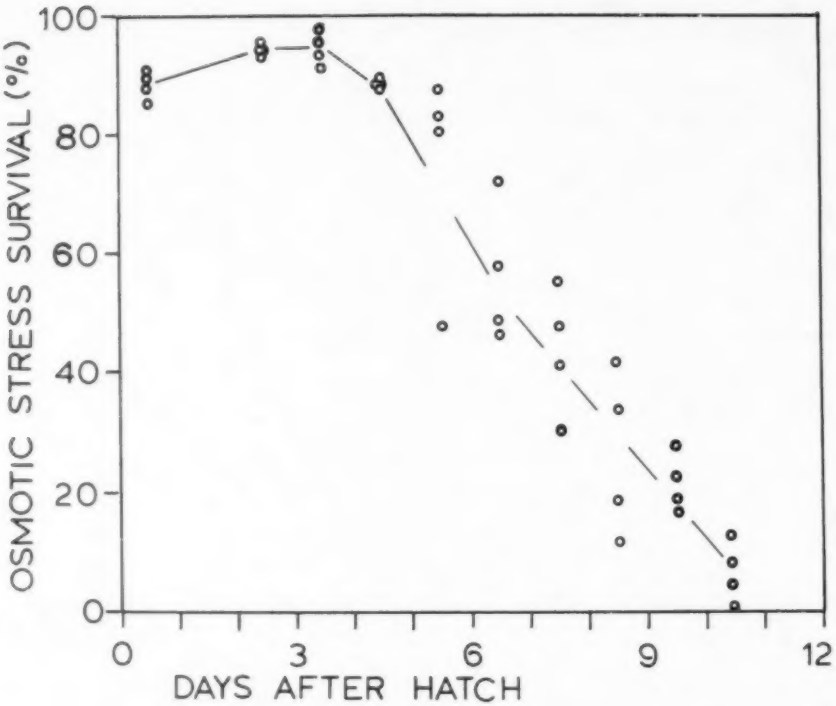

FIG. 3. Osmotic stress survival as a function of age in *C. elegans*. Values represent the percentage of worms surviving 30 minutes of osmotic shock in each sample from four replicate high-density populations. Minimum number of worms in a sample was 50.

DISCUSSION

The underlying difficulty of separating aging, post-reproductive adults from progeny carried by uterine hatch individuals has limited the extent to which biochemical quantities of senescent *C. elegans* can be cultured. Since a spontaneous uterine hatch of a "substantial number of young worms" (Mitchell *et al.*, 1979) exists in the best experimental conditions and noting that a single hermaphrodite can produce 240 offspring with a generation time of 3 to 4 days, it is obvious why past researchers have been limited to approximately 100 hours after hatch for any study of *C. elegans* involving more than a few score of worms. Although *C. elegans* is fecund for only a few days, contamination of the experimental cohort can occur beyond the period of fecundity if uterine hatch exists since the entrained progeny do not emerge as eggs, but hatch, grow, and emerge days later as large juveniles when nothing is left of the parent's interior to consume. The use of mobility to select against uterine hatch allows the study of *C. elegans* during post-reproductive senescence. Our demonstration with *C. elegans* indicates that the inclusion of mobility selection may be a useful technique in the culture of other invertebrates in which the dead or infirm need to be separated from the population.

It should be noted that uterine hatch can be eliminated genetically by the use of strains

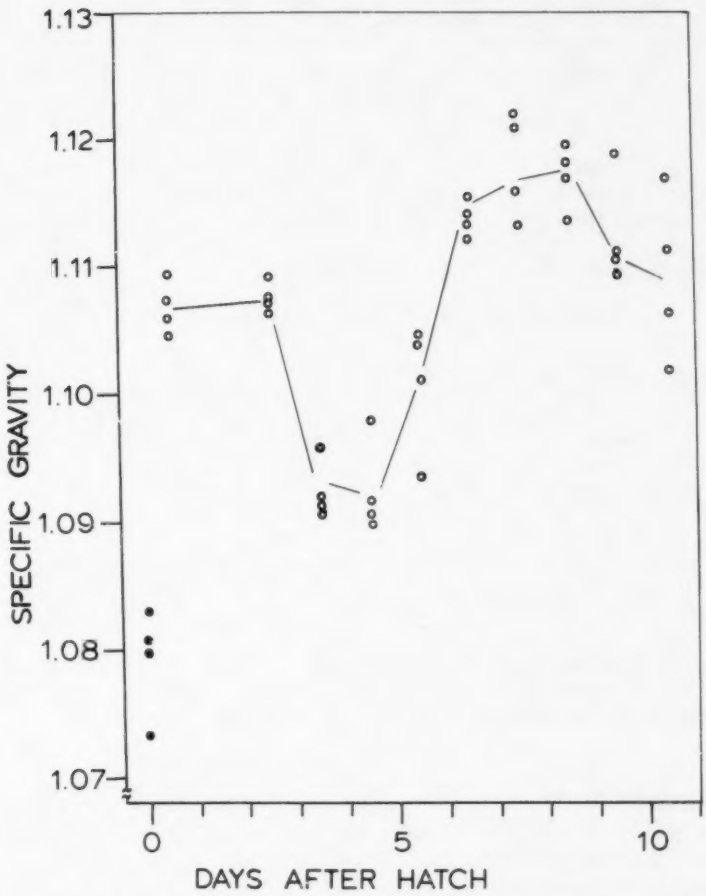

FIG. 4. Specific gravity as a function of age in *C. elegans*. Values represent the mean specific gravity of samples from each of four replicate high-density populations. Values at day 0 represent unhatched eggs with discernable embryos.

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containing temperature-sensitive spermatogensis-inhibition mutations. Unfortunately, the culture of such strains cannot be done at constant temperature (an elevation of temperature is needed to stop spermatogensis) and would not have solved the uterine hatch problem for *C. elegans,* var. Bristol, strain N2, the benchmark variety of *C. elegans*.

Dauerlarva formation, a unique developmental "ageless" stage under study of *C. elegans* (Anderson, 1982), *a priori* should pose a developmental problem to mass culture of *C. elegans* (Cassada & Russell, 1975). But with the inclusion of two pre-synchrony generations and a 12-hour hatch window, the nematodes in the filter/mobility synchrony method develop on the same consistent schedule as other reported nematode systems. The parameters of length, dry weight, and protein per individual worm all indicate typical growing cultures of *C. elegans*. The similarity of survival curves generated by the use of

TABLE 4. SPECIFIC GRAVITY AND AGE OF *C. ELEGANS*.

Time after hatch (days)	*Theoretical sp. gravity*[A]	*Experimental mean specific gravity (n)*[B]	*Age Significant Differences*[C] $\alpha = 0.05$	$\alpha = 0.01$
fertile eggs	?	1.0795 ± 0.0082 (399)		all other days
0.5	1.062	1.1076 ± 0.0078 (1047)	5.5,6.5	3.5,4.5,7.5,8.5
2.5	1.062	1.1068 ± 0.0071 (525)	5.5,6.5	3.5,4.5,7.5,8.5
3.5	1.062	1.0920 ± 0.0073 (468)		0.5,2.5,5.5,6.5,7.5,8.5, 9.5,10.5
4.5	1.063	1.0913 ± 0.0073 (542)		0.5,2.5,5.5,6.5,7.5,8.5, 9.5,10.5
5.5	1.064	1.0993 ± 0.0105 (401)	0.5,2.5	3.5,4.5,6.5,7.5, 8.5,9.5,10.5
6.5	1.068	1.1142 ± 0.0083 (255)	0.5,2.5	3.5,4.5,5.5
7.5	1.075	1.1179 ± 0.0083 (168)		0.5,2.5,3.5,4.5, 5.5,10.5
8.5	1.083	1.1174 ± 0.0107 (302)	10.5	0.5,2.5,3.5,4.5,5.5
9.5	1.094	1.1124 ± 0.0123 (333)		3.5,4.5,5.5
10.5	1.102	1.1083 ± 0.0155 (333)	8.5	3.5,4.5,5.5,7.5
amorphous eggs	?	1.10 – 1.124		

A – Adjusted from data of Zuckerman (1976), Kisiel *et al.*, (1974), no standard deviation given

B – Values express mean ± standard deviation of four pooled cohorts; n represents number of individuals counted at that day.

C – Values express the age (in days after hatch) that the mean specific gravity is significantly different from the mean specific gravity of the examined day, as determined by Duncan's multiple range test. Ages significantly different at both the 5% and 1% confidence levels have not been listed in the $\alpha = 0.05$ for the sake of clarity.

filters at low population density to survival curves generated by other workers using more rigorous and tedious single nematode culture (Bolanowski *et al.*, 1981; Hosono *et al.*, 1982) and the improvement of filter/mobility to filter/settling indicate that the nematodes in filter/mobility synchrony are not atypical.

Specific gravity has been reported to change with age in red blood cells (Dannon & Marikovsky, 1964) and in *Caenorhabditis briggsae* with dramatic increases at the end of reproduction (Zuckerman *et al.*, 1972; Kisiel *et al.*, 1974; Zuckerman, 1974; Zuckerman, 1976; Kisiel & Zuckerman, 1978). Those determinations were conducted with phthalate esters. We question what parameter is changing with age given the visible deformations inflicted upon *C. elegans* in the same length of time as would be necessary for a phthalate specific gravity determination (Figures 5a–f). Perhaps species differences account for the disparity in aging parameters between *C briggsae* and *C. elegans,* for example (Searcy *et al.*, 1976 *v.* Bolanowski *et al.*, 1981). It is our conclusion for free-living nematodes that phthalate esters are inappropriate substances for the determination of specific gravity and that *C. elegans'* changes in specific gravity are dependent on the nature of its gravidity and fecundity.

Early reproductive adults, 3–5 days after hatch, are gravid and fecund, for they contain eggs with distinctly developed and discernable embryos. The lower specific gravity of these adults can be attributed to a loss of a portion of the original mass of the eggs by respiration of the embryos. Later reproductive adults, 6–8 days after hatch, are gravid but non-fecund, and contain eggs with amorphous and granular appearing contents. We

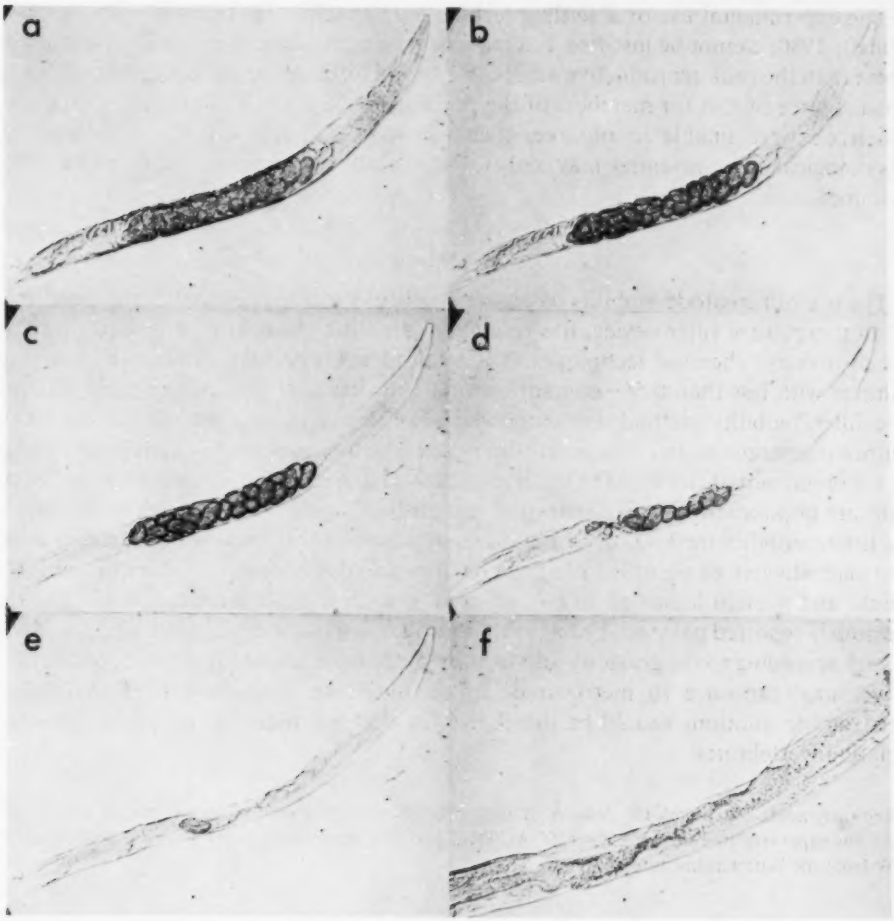

FIG. 5. Time sequence of exposure of a typical 4 day-old *C. elegans* hermaphrodite to a solution of 20% dimethyl phthalate, 80% dibutyl phathalte. Letters indicate exposures of 1, 3, 6, 9, 12, and 14 minutes respectively. All magnifications 200 × except f (closeup of mid-section) 400 ×. Non-movement of nematode apparent within 1 minute of exposure to phthalate ester.

believe these eggs to be either unfertilized or fertilized but non-viable. The higher specific gravity of these adults, similar to that of free unfertilized/non-viable eggs, can be attributed to non-utilization of the original egg mass due to the lack of respiration by the retained unfertilized/non-viable eggs. The previously reported increase in non-fertilization of laid eggs with age in *C. elegans* (Klass, 1977) is consistent with the gravidity/fecundity distinction. We believe this to be the first reported difference in a biophysical parameter, such as specific gravity, between fertile and unfertilized/non-viable eggs in *C. elegans*.

Post-reproductive, senescent adults, more than 9 days after hatch, are non-gravid and non-fecund; they contain no discernable egg forms. The similar specific gravities of these adults and prereproductive individuals can be attributed to the expulsion or reabsorption of the unfertilized/non-viable eggs.

The experimental use of a settling technique to synchronize (Klass, 1977; Bollinger & Willett, 1980) cannot be justified as a mass density separation since the juveniles are more dense than the peak reproductive adults. We found differential settling to be only an additional source of loss for members of the population. If juveniles sink slower than adults, which we were unable to observe, then the source of this difference is most likely physiological, *i.e.,* juveniles may sink slower because they are better or more efficient swimmers.

SUMMARY

The use of nematode mobility as an age synchrony method, as well as the development of an inexpensive filter device, has resulted in a natural model aging system without the use of invasive chemical techniques. The method has been used in both small and large cultures with less than 0.5% contamination of the experimental cohort by its offspring. The filter/mobility method was compared to other methods using the same strain and culture temperature, and the nematodes raised were not measurably different from those raised in individual culture. The method's labor-saving filtration devices allowed for more replicate populations to be examined in the study of aging. As examples of the utility of the filter/mobility method, determinations of various parameters were extended into the post-reproductive phase of the life cycle of *Caenorhabditis elegans.* Individual length, dry weight and protein indicated that *C. elegans'* growth is determinant. Specific gravity, a previously reported parameter of age in nematodes, was dissociated from aging and found to vary according to the gravidity and fertility of the adult and its eggs. The comparison of *C. elegans'* exposure to metrizamide or to phthalate esters indicated that isotonic metrizamide solutions should be the choice for the determination of specific gravity of aquatic invertebrates.

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IRON ACCUMULATION AND LIPID PEROXIDATION IN AGING C57BL/6J MICE

HAROLD R. MASSIE, VALERIE R. AIELLO AND VERA BANZIGER

Masonic Medical Research Laboratory Utica, New York 13503

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Abstract—Total iron concentrations in organs from C57BL/6J male mice increased with age. In animals ranging 45 to 900 days of age liver iron increased by 216%, heart by 66%, kidney by 54% and brain by 27%. Two separate phases of iron accumulation were found in brain, kidney and liver. Between 45–355 days of age brain iron increased by 33% and after 355 days there was no change. For both kidney and liver no change was found until after 355 days of age when liver increased by 140% and kidney by 44%. The kidney and liver results suggest a true aging phenomenon. Liver peroxidation potential as measured by the thiobarbituric acid test tended to increase with age but the differences were not significant. The addition of metal ion chelators greatly reduced lipid peroxide values for all organs but no significant age-related trend was evident. We conclude that the large aging-related increases in tissue iron are not correlated with increased concentrations of lipid peroxides.

INTRODUCTION

SINCE HARMAN (1956) first proposed the free radical theory of aging, many investigators have been interested in the possibility that aging may involve a process whereby the polyunsaturated fatty acids of cell structures undergo a free-radical-initiated peroxidation. Although products of this reaction should be more concentrated in older organisms, surprisingly few measurements of the products of peroxidation versus age have been reported and not all of these have been consistent. Yoshikawa and Hirai (1967) have reported an increase with age in the concentration of malonyldialdehyde, a known product of lipid peroxidation, in the brains of Wistar rats. On the other hand, Grinna and Barber (1973) found a considerable decrease with age in the concentration of malonyldialdehyde in male Sprague-Dawley rats. Armstrong *et al.* (1978) found an increased concentration of peroxides with age in *Drosophila* fruit flies.

Lipid peroxidation is known to be catalyzed by iron and iron complexes (see Aust and Svingen [1982] for an excellent review). It is possible, therefore, that changes in the iron content of various organs with age might greatly influence their susceptibility to lipid peroxidation. This could be especially significant when using the thiobarbituric acid test since it involves incubating the analytical sample prior to the actual measurement. Small changes in iron concentration with age might, therefore, produce anomalously high peroxide values. In this communication we report both iron concentration and lipid

peroxidation as measured by the thiobarbituric acid test in liver, kidney, heart and brain from mice of different ages.

MATERIALS AND METHODS

Peroxidation

Lipid peroxidation was measured by the thiobarbituric acid test as modified by Uchiyama and Mihara (1978). This test measured the amount of malonyldialdehyde, a decomposition product of lipohydroperoxides, in tissue homogenates. Organs were removed from C57BL/6J male mice after cervical dislocation between 9:00 A.M. and 11:00 A.M. (EST). Heart, liver, kidney and brain were perfused with 0.1M HEPES buffer (pH 7.8) and then homogenized with a 30 ml teflon-glass Thomas Tissue Grinder (Philadelphia, PA) in cold (4°C) 1.15% KCl to make a 10% whole homogenate (it was necessary to use a glass on glass homogenizer for heart). Heating time for the analysis was 45 min at 100°C and absorbance of the butanol extract was read at 535 and 520 nm. The difference was taken as the thiobarbituric acid (TBA) value and was expressed as absorbance per mg of dry weight. Samples were analyzed immediately after homogenization. We found that storing the samples prior to analysis yielded higher and erratic TBA values.

Iron

Iron was determined by atomic absorption on a Varian 1250 spectrophotometer with a carbon-rod atomizer Model 90. Single organs were placed on acid-washed microscope slides and dried overnight at 88°C. Whole organs were digested in Ultrex HNO_3 (J.T. Baker Chemical Co.) and kept for 5 days at room temperature. The fat layer formed on top of the liver samples was removed by aspiration. Iron was expressed as nanograms of iron per milligram of dry weight.

Animals

Male C57BL/6J mice obtained from Jackson Labs., Bar Harbor, Maine were used for all experiments. Mice were purchased at one month of age and introduced into our colony. The median survival time in our colony is usually about 900 days and the maximum seldom exceeds 1000 days. Purina laboratory chow and tap water were given *ad libitum*. The age groups used were 37–69, 209–218, 636–645, and 818–888 days of age.

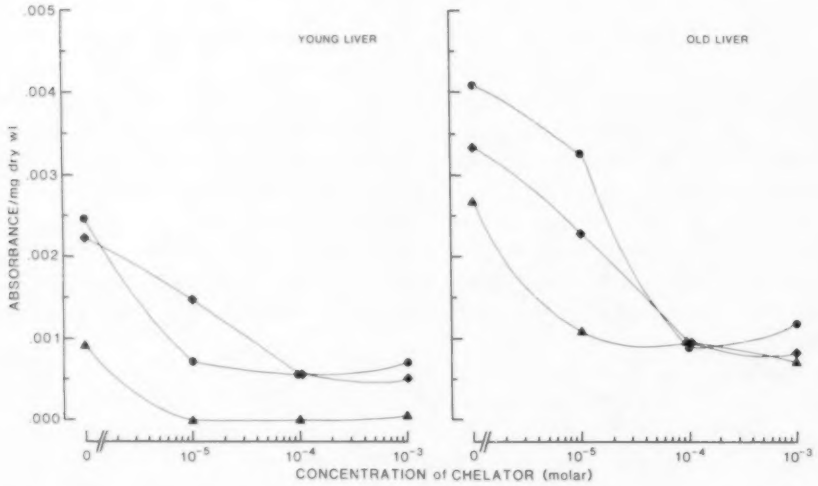

FIG. 1. Influence of metal ion chelators on lipid peroxide content of liver homogenates from young (37–69 day old) and old (882–888 day old) male C57BL/6J mice. Peroxidation by the TBA method is expressed as absorbance difference between 535 and 520 nm per milligram of homogenate dry weight. ●, EDTA; ◆, CDTA; ▲, DTPA. Concentrations are final chelator concentrations in the reaction mixture.

RESULTS

The possible involvement of metal ions in lipid peroxidation is indicated in Figure 1. Liver homogenates from both young and old mice were greatly affected by three different metal ion chelators, EDTA (ethylenediaminetetraacetic acid), CDTA (1,2-cyclohexanediaminetetraacetic acid) and DTPA (diethylenetriaminepentaacetic acid). Maximum inhibition of color development in the TBA test occurred at 10^{-4} M for all three chelators. Also, these chelators gave approximately the same degree of inhibition in liver, kidney, heart and brain. In young liver 10^{-5} M DTPA completely inhibited the reaction. In both young and old heart the TBA reaction was completely inhibited by 10^{-5} M EDTA. These results suggested that lipid peroxidation potential might change during senescence simply as a result of changes in metal ion concentrations.

We, therefore, analyzed whole homogenates with and without added chelator. Since EDTA gave the most consistent inhibition for all four organs it was used at a concentration of 10^{-4} M. The data for liver are shown in Figure 2. In the absence of chelator, TBA values appeared to increase with age but the least squares analysis of the data showed no significant age-related change (Table 1). The values for liver plus chelator were greatly

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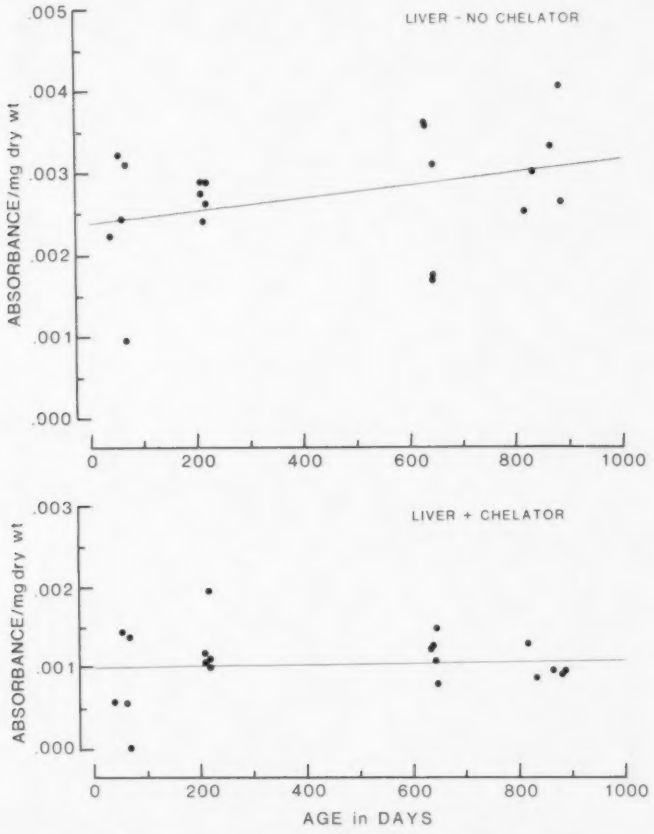

FIG. 2. Lipid peroxide content versus age for whole liver homogenates of male C57BL/6J mice when prepared in the absence and presence of EDTA (10^{-4}M final concentration). Each point represents a different mouse.

TABLE 1. LEAST SQUARES EQUATIONS FOR TBA VALUES

Sample	*Equation*	*N*	*Correlation Coefficient*	*Degree of Certainty*
Liver	TBA = $+7.56 \times 10^{-7}$Age + 0.0024	20	0.343	80–90%
Liver + EDTA	TBA = $+1.24 \times 10^{-7}$Age + 0.0010	20	0.101	<80%
Kidney	TBA = $+5.63 \times 10^{-7}$Age + 0.0049	20	0.143	<80%
Kidney + EDTA	TBA = -1.51×10^{-7}Age + 0.0020	20	−0.147	<80%
Brain	TBA = $+2.71 \times 10^{-7}$Age + 0.0078	20	0.048	<80%
Brain + EDTA	TBA = -1.50×10^{-7}Age + 0.0029	20	−0.084	<80%
Heart	TBA = $+5.78 \times 10^{-7}$Age + 0.0046	20	0.099	<80%
Heart + EDTA	TBA = -8.53×10^{-7}Age + 0.0022	20	−0.212	<80%

Least squares equations for TBA values versus age for organs from male C57BL/6J mice. Age is expressed in days and TBA as absorbance/mg dry wt. N is the number of animals used. For the EDTA samples, EDTA was at a concentration of 10^{-4}M in the reaction mixture. Five animals were used for each age group, 37–69, 209–218, 636–646, and 818–888 days of age.

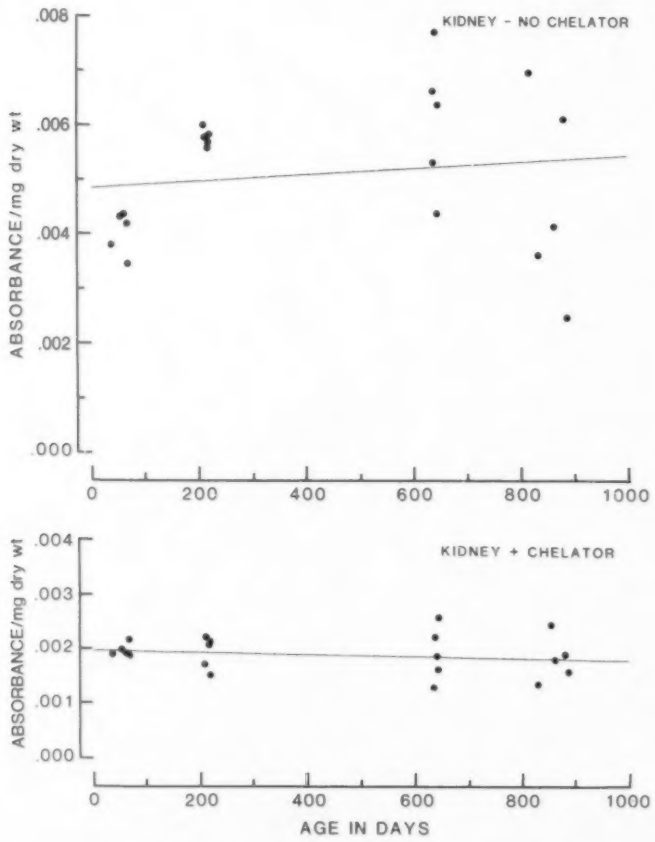

FIG. 3. Lipid peroxide content versus age for whole kidney homogenates in the absence and presence of EDTA.

reduced and no age-related change was observed (Figure 2). For kidney, heart and brain in the absence of chelator we also found an age-related increase in TBA values. In the presence of chelator the values were drastically reduced and all decreased with age. However, none of the changes with or without chelator were significant (Table 1).

Since iron is known to be one of the most significant catalysts for lipid peroxidation and also exists in most tissues at relatively high concentrations, it seemed desirable to measure the age-related change in total iron for all four organs. We found large and highly significant changes in all organs. Least squares fit lines are drawn through the data in Figures 6–9. Liver showed the largest accumulation of iron with age with a change of 216% up to 900 days of age. Heart increased by 66%, kidney by 54% and brain by 27%.

The data for liver iron for ages 45 to 888 days gave a least squares equation of Iron = 0.372 Age + 154, with iron expressed as nanograms of iron per milligram of dry

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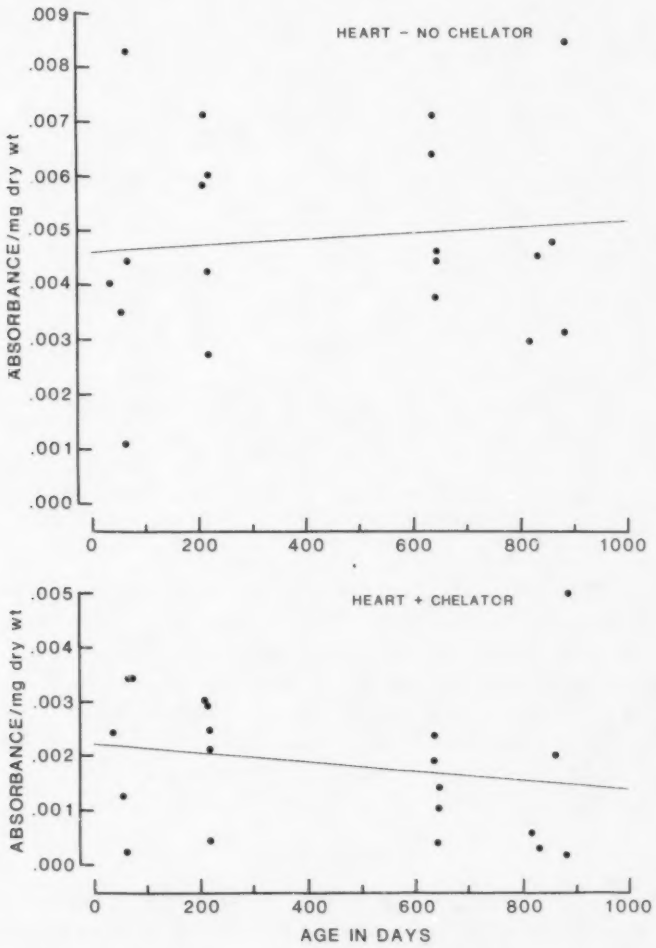

FIG. 4. Lipid peroxide content versus age for whole heart homogenates in the absence and presence of EDTA.

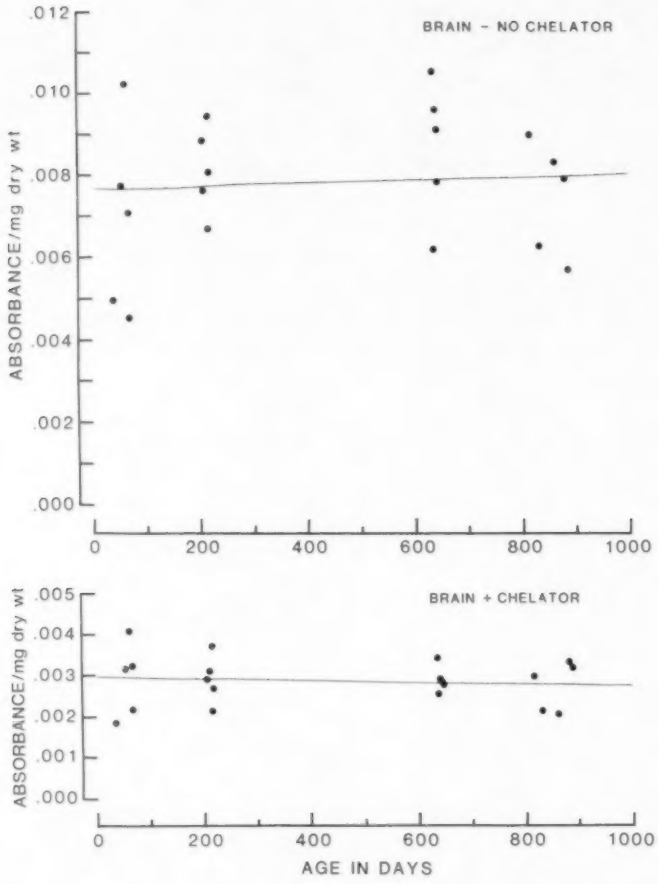

FIG. 5. Lipid peroxide content versus age for whole brain homogenates in the absence and presence of EDTA.

weight and age in days. It is clear from Figure 6, however, that there was no change in hepatic iron between 45 and 355 days of age. The increase in hepatic iron began at 355 days of age and gave a least squares equation of Iron = 0.587 Age + 15.5.

The increase of heart iron with age was significant when all data were considered (Figure 4). However, when the three high values at 777 days of age were removed, the increase was no longer significant ($0.1 < p < 0.2$), with Iron = 0.077 Age + 291. Although we have no reason to justify this removal it would be prudent to consider that no increase with age occurs in heart iron.

DISCUSSION

The iron content of liver, heart, kidney and brain increased with age in C57BL/6J mice. The greatest change occurred in liver with the increase in iron content beginning after 355 days of age and after growth had been completed. Whether or not this is a general phenomenon in other organisms or can be implicated in senescence remains to be seen.

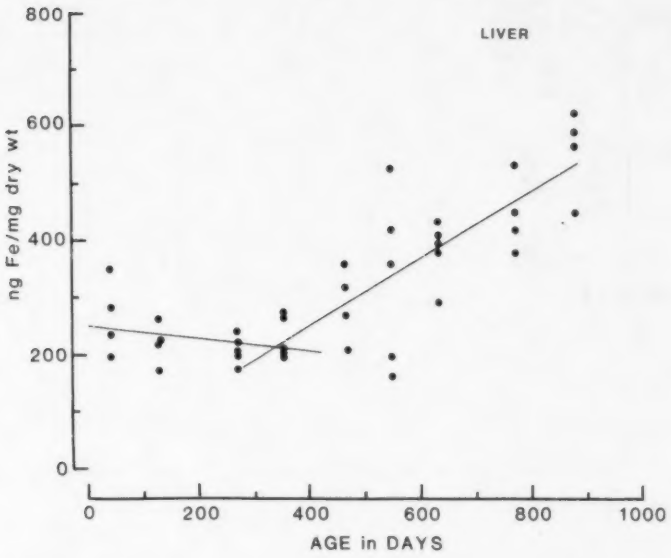

FIG. 6. Iron content versus age for liver from C57BL/6J male mice. Least squares equation: Iron = 0.587 Age + 15.5 where iron is expressed as nanograms of iron per milligram of dry weight and age is in days. Equation is for animals between 355 and 886 days of age. Number of mice (N) = 27; $p < 0.001$.

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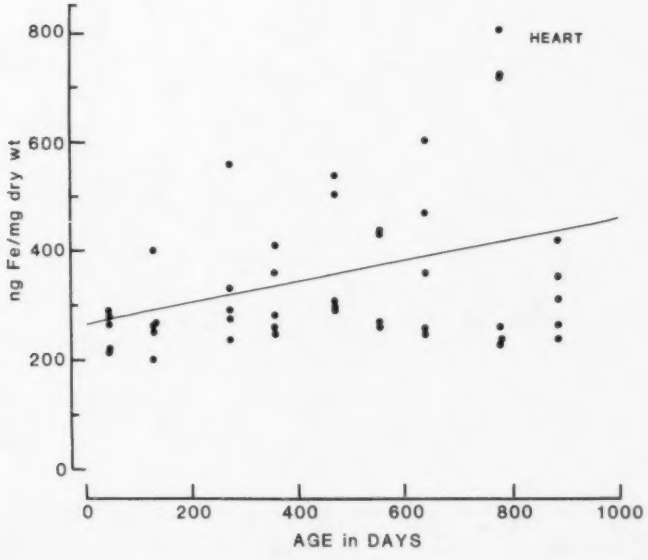

FIG. 7. Iron content versus age for heart from C57BL/6J male mice. Iron = 0.196 Age + 264; N = 44; $0.01 < p < 0.05$.

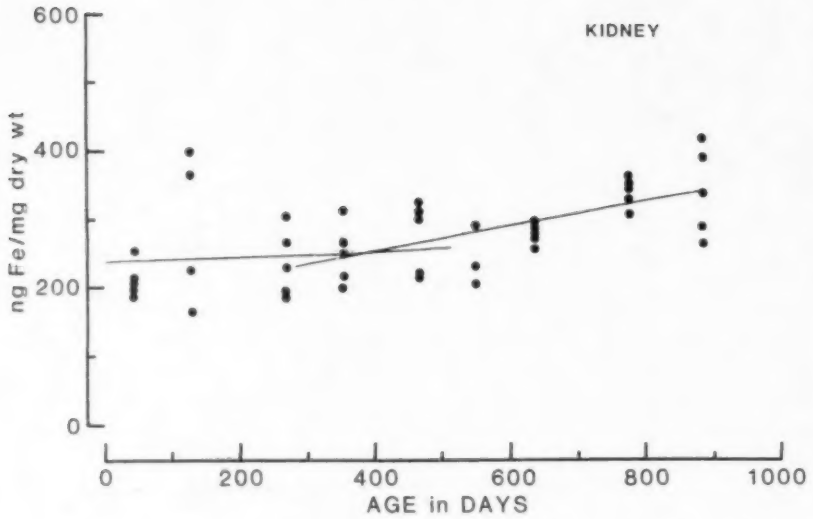

FIG. 8. Iron content versus age for kidney from C57BL/6J male mice. Iron = 0.189 Age + 175; N = 28; $p < 0.001$; for ages 355–886 days.

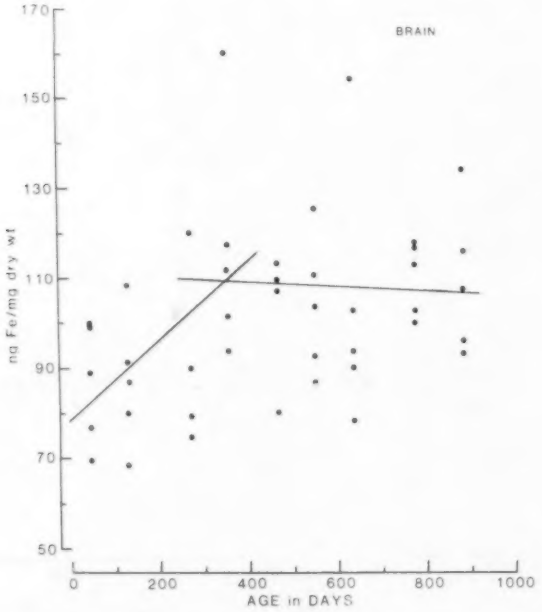

FIG. 9. Iron content versus age for brain from C57BL/6J male mice. Iron = 0.088 Age + 78.6; N = 19; $0.01 < p < 0.05$; for ages 45–355 days.

Another result is that lipid peroxidation potential as measured by the thiobarbituric acid test did not significantly increase with age in any organ tested. This result was surprising since it has been widely assumed that lipid peroxidation is probably involved in the aging process. Thus we are unable to support the results of either Yoshikawa and Hirai (1967) who found an increase with age or Grinna and Barber (1973) who found a decrease with age. Perhaps dietary factors such as vitamin E or selenium are responsible for these different results. Our results with chelators suggest another possibility, that metal ion concentrations play a major role in determining the level of peroxides found in any tissue homogenate.

Of all the possible contributors to lipid peroxidation in a whole homogenate system, one would expect that an increase in iron concentration would lead to greater concentrations of lipid peroxides. We did not find this to be true. One explanation could be that most of the iron is in the ferric oxidation state and, therefore, less likely to catalyze lipid peroxidation. The presence of iron chelators or oxidizing agents may change with age. Peroxidation inhibitors as described by Grinna and Barber (1973) may also be present. Whatever the reason it is clear that a large increase in tissue iron with age does not necessarily lead to higher levels of lipid peroxidation. Other molecular populations, however, may be altered as a result of age-related changes in iron content and thereby influence senescence in as yet unknown ways.

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SUMMARY

Iron concentrations in organs from C57BL/6J male mice increase with age. In animals ranging 45 to 900 days of age liver iron increased by 216%, heart by 66%, kidney by 54% and brain by 27%.

Lipid peroxidation potential as measured by the thiobarbituric acid test tended to increase with age in all organs but the differences were not significant. The addition of metal ion chelators greatly lowered lipid peroxide values for all organs but no significant age-related trend was evident.

We conclude that the large age-related increase in tissue iron does not lead to greater concentrations of lipid peroxides.

Acknowledgments—We wish to thank Drs. Malcolm Baird and Arthur Iodice for their helpful criticism.

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INCREASED INTESTINAL ABSORPTION OF OLEIC ACID WITH AGING IN THE RAT

DANIEL HOLLANDER, M.D., and VIOLETTA D. DADUFALZA, M.S.

Departments of Medicine, Physiology and Biophysics, University of California, Irvine
Long Beach Veterans Administration Medical Center, Long Beach, California

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Abstract—Changes in nutrient absorption could be responsible for some of the disorders associated with aging. Oleic acid is the most common dietary fatty acid. Therefore, we investigated its absorption by the small intestine of aging rats *in vivo*. We used a single pass intestinal perfusion technique to study absorption in animals between 6 and 138 weeks of age. Rats less than 70 weeks of age absorbed 14,000 to 15,000 nmol of oleic acid per 100 cm/hr. In contrast, rats 94 weeks of age or older, absorbed 22,000 to 23,000 nmol of oleic acid per 100cm/hr. The 61% increase in oleic acid absorption as the rats aged correlated with a decrease in the resistance of the unstirred water layer from 0.8 to 0.5 min/cm³/100cm. Concomitantly, the surface area of the unstirred water layer increased from 97.1 to 141.2 cm²/100cm. The changes in unstirred water layer resistance and surface area explain the observed increase in absorption of oleic acid with aging. The increased surface area and decreased resistance of the unstirred water layer are both conducive to increased intestinal absorption of this lipid nutrient as the animals aged.

Keywords: Aging, Intestinal Absorption, Fatty Acids, Unstirred Water Layer

INTRODUCTION

OLEIC ACID is a common dietary fatty acid in man. It is ingested in a triglyceride form (Triolein) and hydrolized prior to its intestinal absorption to free oleic acid and monoolein (Greenberger, Rodgers, and Isselbacher, 1966).

The influence of aging on intestinal functions, intestinal absorptive capacity and dimensions of the unstirred water layer are not well understood. Recent information in animals indicates that aging is associated with changes in the intestinal absorption of cholesterol (Hollander and Morgan, (a), 1979), vitamin A (Hollander and Morgan, (b), 1979), amino acid (Winter *et al.*, 1971) and glucose (Thomsons, 1979). The nutritional importance of oleic acid and the lack of information regarding the influence of aging on its absorption led us to investigate its intestinal absorption in the unanesthetized restrained animal.

Address reprint requests to: Daniel Hollander, M.D. Medical Science I, Room C-340 University of California, Irvine, CA 92717

MATERIALS AND METHODS

Animals

Male, Sprague-Dawley rats were purchased as weanlings (Charles River Laboratories, Willmington, MA) and raised in our aging animal colony. The animals had free access to water and rat feed (Lab-blox A 8604-00, Allied Mills Inc., Chicago, IL) and were not fasted prior to experimentation. Rats varying in age from 6 to 138 weeks were used for this series of experiments. The animals were chosen at random from the colony with 3–6 animals studied at each age group.

Materials

9,10-^{3}H oleic acid (New England Nuclear Corp, Boston, MA, specific activity 10.0 ci/mmol) was used as a tracer compound. Thin layer chromatography on silica gel developed in hexane; diethyl-ether; acetic acid (70:30:1) demonstrated the radiochemical purity of the compound to be >99%. Carrier oleic acid (Sigma Chemical Co., St Louis, MO) had less than 1% impurities. ^{14}C-Inulin (New England Nuclear, Boston, MA; specific activity 2.5 mCi per gram; radiochemical purity >99%) was used as a non-absorbable marker (Esposito and Csaky, 1974). Recrystalized sodium taurocholate (Calbiochem Co, San Diego, CA) was found by thin layer chromatography (Gregg, 1966) to have less than 1% impurities. Sodium hydrogen phosphate and disodium hydrogen phosphate (Fisher Scientific Co.) were used as components of the Krebs phosphate buffer (Umbreit, Burris, and Stauffer, 1972).

Intestinal perfusate

The standard perfusate solution contained: oleic acid (2mM), sodium taurocholate (10mM), tracer ^{3}H oleic acid and ^{14}C inulin in the Krebs phosphate buffer base, pH 6.5. The solution was sonicated for 5 minutes at 70 watts of power to form micelles. The integrity of the micelles and the distribution of oleic acid between the micellar particles (>95%) and free monomeric form were established by standard techniques (Hollander and Muralidhara, 1977)

Experimental methods

The animals were prepared for perfusion by methods we have described previously (Hollander, 1981). The animal was anesthetized with ether and a 30cm segment of its proximal jejunum was cannulated. Its abdominal cavity was then closed and the animal was allowed to recuperate from anesthesia before it was placed in a plexiglass restraining cage. The animal's body temperature was maintained at 37°C by methods described earlier (Hollander, 1980). A syringe pump (Sage model 351, Orion Research Inc, Cambridge, MA) was used to infuse the micellar solution at a constant rate of 0.5 ml/min. After an initial 10 minute perfusion period, the outflow was collected in six 10 minute aliquots for a period of 60 minutes. At the completion of the experiments the animals were killed with an overdose of ether. The perfused segment was removed and dried for 48 hours with a 5-gram weight suspended at the dependent end of the segment to ensure constant degree of stretching of the bowel.

Radioactivity determination

Duplicate 100-μl aliquots were taken from each 10 minute collection and radioactivity counted in a liquid scintillation counter with automatic quench calibration at ambient temperature (Beckman LS-250, Beckman Instruments, Fullerton, CA). The two radiosotopes were seperated by the sequential additions of internal standards with mathematical calculation of individual isotope contribution.

Calculation and statistical analysis

The use of inulin as a non-absorbable marker (Esposito and Csaky, 1974) enabled us to correct the absorption rates for fluid shifts. Absorption of oleic acid was then calculated from its rate of disappearance from the lumen (Hollander, 1981). Oleic acid absorption by rats of various ages was compared statistically by using Student's t-test (Draper and Smith, 1966) and analysis of variance (Dixon and Massey, 1969).

Dimensions of the unstirred water layer (UWL):

The thickness of the UWL was measured according to the method of Diamond (Diamond, 1966). Briefly, changes in the transmucosal potential difference (PD) were induced by rapid changes in the intraluminal sodium concentration (154 to 25mM NaCl); meanwhile, the osmolarity of the solution was kept constant with mannitol.

The oleic acid absorption data were analyzed according to the equations derived by Westergaard and Dietschy (Westergaard and Dietschy, 1974) in order to calculate the surface area (Sw) and resistance (R) of the UWL. The UWL surface area Sw = (Jd) (d)/C_1) (D) where (Jd) is the observed rate of oleic acid absorption at various ages, (d) is the thickness of the UWL as determined by the change in PD according to the Diamond method (Diamond. 1966), (C_1) is the concentration of oleic acid in the perfusate and (D) is the diffusion coefficient of oleic acid. Once the surface area (Sw) of the UWL was determined, the resistance (R) to diffusion of oleic acid across the UWL was calculated from the equation R = (d)/(Sw) (D) as derived by Westergaard and Dietschy (Westergaard and Dietschy, 1974).

RESULTS

The absorption rates of oleic acid were determined in six different age groups animals varying in age from 6 to 138 weeks. Each animal contributed six different absorption periods for analysis. The coefficient of variation between absorption rates at different time periods for each animal was less than 10%. For the final analysis, all the individual 10 minute absorption rates from all animals at each specific age group were pooled and a final rate of absorption was calculated and expressed as a mean ± standard error.

The animals gained weight up to 52 weeks of age; thereafter their body weight was stable. The intestinal length increased up to 52 weeks of age when this parameter stabilized and increased no further (Table 1). Although we did not measure the intestinal cylindrical surface area, our previous work with animals from the same colony indicated that the surface area does not increase in our animals after six weeks of age and that the intestinal surface area remains proportional to the intestinal length (Meshkinpour, Smith, and Hollander, 1981). The mean oleic acid absorption rates of animals up to 70 weeks of age varied from 13,444 to 15,481 nmol/100 cm/hr. As the animals aged, the absorption rate increased to a maximal rate of 22,920 nmol/100 cm/hr at 138 weeks of age (Table 2).

DISCUSSION

The absorption rate of oleic acid by the unanesthetized rat increased by 61% in the latter half of the life span of the animals. During the first half of the life span of the animals (70 weeks of age or less) the absorption rate of oleic acid remained between 13,444 to 15,481 nmol/100cm/hr. In the latter half of the life span of the animals, absorption rate increased to 22,920 nmol/100cm/hr (Table 2). At first glance, an increase in absorption by 8,000 nmol/100cm/hr may not seem to be biologically significant. However, if this increase is multiplied by the four or more hours of absorption per day and is further multiplied by the year or more of the animal's remaining lifespan, the increase in lipid absorption becomes biologically significant.

TABLE 1. ANIMAL PARAMETERS

Age (weeks)	*Number of Animals*	*Animal Weight (gm)*	*Intestinal Dry Weight (gm)*	*Intestinal Length (cm)*
6	6	187.3 ± 9.3	1.56 ± .06	109.5 ± 4.6
22	6	572.1 ± 20.4	2.16 ± .09	111.5 ± 2.7
52	4	776.3 ± 54.5	2.56 ± .27	137.0 ± 4.6
70	4	753.3 ± 21.6	2.16 ± .13	136.3 ± 5.3
94	3	764.5 ± 76.6	4.70 ± .18	128.2 ± 4.1
138	3	775.0 ± 58.9	5.51 ± .43	136.3 ± 5.5

Values are mean ± S.E.

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TABLE 2. OLEIC ACID ABSORPTION

Age (weeks)	*Number of Animals*	*Absorption (nmol/100cm/hour)*
6	6	14419.3 ± 505.4
22	6	14941.7 ± 860.8
52	4	13444.8 ± 1165.9
70	4	15481.5 ± 1489.5
94	3	22479.0 ± 1141.2*
138	3	22920.9 ± 2033.7*

*$p < 0.05$ when compared to baseline absorption rate at 6 weeks of age.
Values are mean ± S.E.

Since the composition of the intestinal perfusate was artificially controlled and since endogenous biliary and pancreatic secretions were excluded from the perfused intestinal segment, age-related changes in biliary or pancreatic secretions cannot account for the increase in oleic acid absorption as the animals aged. The increase in oleic acid absorption by the aging animals cannot be caused by an increase in their intestinal surface area either because the intestinal surface area of animals from our colony did not increase after six weeks of age (Meshkinpour, Smith, and Hollander, 1981).

Theoretically, age-related changes in the unstirred water layer, namely, an increase in its surface area or a decrease in its resistance or thickness, could account for the increase in oleic acid absorption in the aging animals. To test this hypothesis we calculated the surface area and the resistance of the unstirred water layer (UWL) in our animals using standard formulas (Westergaard and Dietschy, 1974). We found that the resistance of the UWL started to decrease after 52 weeks of age (Table 3). The highest value of the UWL resistance (0.8 min/cm^3/100 cm) was found in animals six weeks old and its lowest (0.5 min/cm^3/100 cm) in animals 138 weeks old. These findings indicate that the lowered resistance to diffusion of oleic acid across the UWL as the animals aged is one mechanism partially responsible for our observations. Another explanation is that the total surface area (Sw) of the UWL increased as the animals aged (Table 3). The Sw at six weeks of age was 97.1 while at 138 weeks of age it increased to 141.2 cm^2/100 cm. Since the intestinal length is proportional to the intestinal cylindrical surface area (Meshkinpour, Smith, and Hollander, 1981), and since the intestinal cylindrical surface area (Sm) remains relatively

TABLE 3. INFLUENCE OF AGING ON THE SURFACE AREA (Sw) AND RESISTANCE (R) OF UNSTIRRED WATER LAYER (UWL)

Age (Weeks)	*UWL Surface Area* $Sw = (jd)(d)/(C_j)(D)$ *(cm^2/100 cm length)*	*Resistance of UWL* $R = (d)/(Sw)(D)$ *(min/cm^3/100cm)*
6	97.1	0.8
22	100.7	0.8
52	90.6	0.8
70	95.4	0.7
94	138.5	0.5
138	141.2	0.5

TABLE 4. INTESTINAL SURFACE AREA (SM), UNSTIRRED WATER LAYER SURFACE AREA (SW) AND THEIR RELATIONSHIP AS THE RATS AGED

Age (Weeks)	*Intestinal Surface Area (Sm) (cm^2/100 cm)*	*UWL Surface Area (Sw) (cm^2/100 cm)*	*Sw / Sm*
6	124.5	97.1	0.78
22	131.4	100.7	0.77
52	118.9	90.6	0.76
70	127.6	95.4	0.75
94	119.0	138.5	1.16
138	120.7	141.2	1.17

constant as the animals age (Meshkinpour, Smith, and Hollander, 1981), the ratio of Sw to Sm was found to increase in our animals as they aged (Table 4). The increased ratio of Sw to Sm provides a greater surface area for absorption of oleic acid by the intestine of older animals and can additionally account for the increase in absorption of oleic acid in older animals (Table 2).

The present studies showing increased absorption of oleic acid with aging and earlier studies showing similar findings with cholesterol (Hollander and Morgan, (a), 1979) and vitamin A (Hollander and Morgan, (b), 1979) indicate that absorption of some lipids increases in the latter portion of the life span of animals. Whether similar changes occur in man is unknown, but if they do the implications would be important for the absorption of both dietary lipids and lipid-soluble drugs or carcinogens. If the absorption of lipid nutrients and lipid-soluble drugs increases in aging individuals, this age-related change in absorption could partially explain the prevalence of lipid related cardiovascular disorders and increased sensitivity to lipid soluble drugs in aging individuals.

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TISSUE-SPECIFICITY OF REACTIVITY TO CONCANAVALIN-A OF HUMAN CELLS IN CULTURE

SADAYUKI BAN*[1],† and SHOZO IIDA†

Department of Pathology* and Clinical Laboratories†,
Radiation Effects Research Foundation, Hiroshima 730, Japan

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Abstract—Concanavalin-A (Con-A) reactivity was studied to identify the tissue-specificity of cells established from various normal human tissues. Cells were treated with Con-A-labelled human red blood cells (C-RBC). C-RBC was not adsorbed on the cells derived from the bone marrow, skin and liver. Lung-derived fibroblast cells showed weak C-RBC adsorption. Kidney-derived cells showed epithelial morphology and easily adsorbed C-RBC. These suggest that a large number of Con-A receptors exists on the membrane surface of kidney cells.

INTRODUCTION

ESTABLISHMENT OF a simple method for identifying the neoplastic nature of cultured cells is important for the advancement of cancer research. The indices of neoplastic transformations most frequently used at present include: *a*) loss of post-confluent inhibition of cell proliferation (Holley and Kierman, 1968; Pollack *et al.*, 1968); *b*) proliferative ability in soft agar (MacPherson, 1963; Freedman and Shin, 1974; Tucker *et al.*, 1977; Sanford *et al.*, 1967; Persky, 1982); *c*) cellular immortality (Igel *et al.*, 1975); *d*) loss of diploidy (Sumner, 1981; Trent and Salmon, 1981; Yunis, 1981); and *e*) changes in Con-A reactivity (Oishi *et al.*, 1981). Of these, examining changes in the reactivity of cells to concanavalin-A (Con-A) by adsorbing red blood cells treated with Con-A (abbreviated as C-RBC) (Con-A hemadsorption test) has been employed because of the simplicity of the procedure and the specificity for neoplastic transformation is high. The method is especially useful to detect tumor-associated characteristics of fibroblasts easily obtained in culture.

Recently, Aizawa *et al.* (1980 a,b) succeeded in employing C-RBC adsorption as an index for the aging of cells *in vitro*. On examining C-RBC adsorption of cultured fibroblasts derived from normal human fetal lungs, they found that absorption was higher in older cells than in young cells. The number of C-RBC adsorbed by the cells was proportional to the age-related increase of the cell membrane surface area. There was no change in the number of Con-A receptors per unit area of the membrane surface of the cells used in their study.

We examined whether C-RBC adsorption of the membrane surface of cultured human

[1]Present address: Biology Department, Brookhaven National Laboratory, NY 11973.
Address all correspondence to Dr. S. Ban.

cells had tissue-specificity. Cells established from tissue samples of lung, liver, skin, and bone-marrow had a fibroblast morphology (or fibroblast-like cells) and hardly adsorbed any C-RBC. However, most of the cells established from the kidney showed an epithelial morphology with very strong C-RBC adsorption.

Materials used in this study were obtained from organs which had been confirmed at autopsy to have neither tumors nor degeneration.

METHODS AND PROCEDURE

Tissue and cells

All cells used in this study were established in our laboratory and were obtained from resected tissue during autopsies. Tissue sections were washed thoroughly in a buffer solution containing 500 μg/ml of streptomycin and 500 U/ml of penicillin. These sections were minced into 1 mm^3 or smaller pieces in 5 cm plastic dishes (Falcon Co. USA) containing a small amount of culture solution. The small pieces were put into Eagle's MEM culture solution (Nissui Pharmaceuticals, Tokyo) containing fetal bovine serum (Hy-Clone Co. USA), and cultured at 37°C in 5% Co_2-95% air. After 3–10 days, the culture medium was replaced with Eagle's MEM medium containing 10% fetal bovine serum. When numerous cells began to proliferate from the pieces of tissue, subcultures were initiated according to methods previously reported (Ban *et al.*, 1980 a, 1982). Eagle's MEM supplemented with 10% fetal bovine serum was used for the maintenance and subculture of cells. A solution containing 0.125% trypsin (Difco, USA) and 0.01% EDTA (Wako Chemicals, Tokyo) was used for cell harvesting. Figure 1 shows the tissues used and the ages of the donors.

Preparation of Con-A-labelled red blood cells (C-RBC)

The method of Oishi *et al.* (1981) was employed in the preparation of Con-A labelled RBC. Two ml of human male blood (type A or type O) was collected by venepuncture. After suspending in 5 ml of PBS (−), the sample was washed twice by centrifugation for five minutes at 1,000 rpm. Then 0.03 ml of the red blood precipitate was added to 3 ml of PBS, in which concanavalin-A (Con-A; Sigma, USA) had been dissolved at various concentrations. Cells and Con-A were allowed to react at 37°C for 30 minutes. After reaction, the red blood cells were washed and centrifuged twice, and 3 ml of the PBS (−) was dispensed into each test tube. The C-RBC were freshly prepared for each experiment.

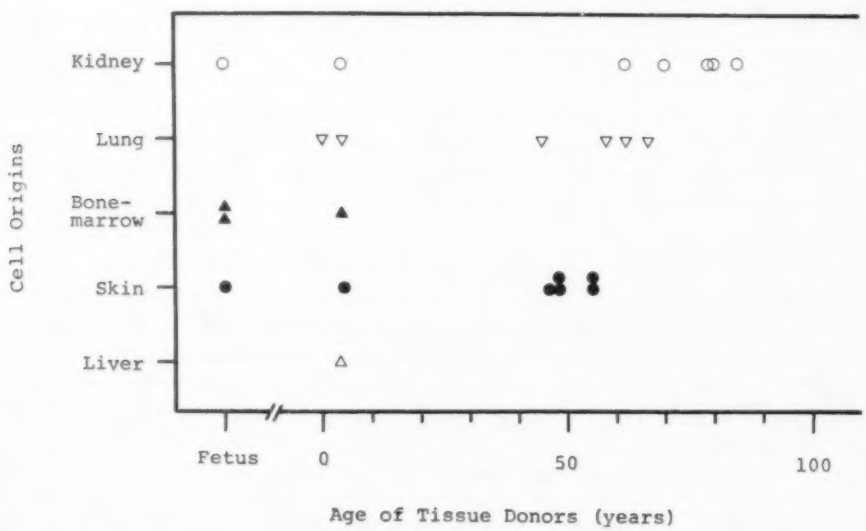

FIG. 1. Origin of established cells and ages of cell donors. Each organ was confirmed to have neither tumor nor cellular degeneration at the time of the autopsy.

Con-A hemadsorption test

For all cell types, the fifth to the tenth generation were used in the tests. The target cells were cultured in advance for two days in αMEM culture solution supplemented with 10% fetal bovine serum. 2.5×10^3 cells were inoculated in wells with a growing surface area of 1.6 cm^2 (Lab-Tek, USA), and cultured overnight at 37°C in 5% CO_2. After the cells were washed twice in PBS (+), 1 ml of C-RBC was added to each well. After the mixture was allowed to react at 37°C for 10 minutes, the cells were washed three times in PBS (+). If C-RBC remained on the surface of the culture chamber, the washing procedure was repeated. Before the cells dried, they were fixed for three minutes in aceton-formalin fixative (pH 6.6). After washed in water, they were stained in Giemsa's staining solution, and enclosed with a cover glass. Vermilion-colored human red cells were adsorbed on cells stained with Giemsa. The number of C-RBC adsorbed on the surface of the 200 or more cells in each slide was counted under a microscope.

RESULTS

Figure 2 shows the number and frequency of C-RBC adsorbed per cell using various organ derived cells obtained from the same individual (boy four years old).

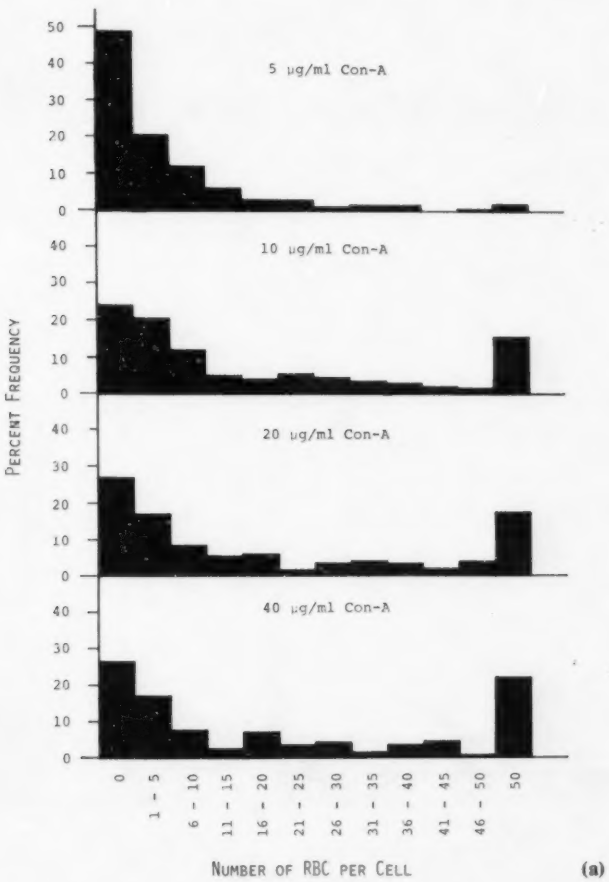

FIG. 2. Distribution of C-RBC adsorption (human red blood cells labelled with Con-A) by culture cells established from the various organs of a four-year-old boy. Concentrations of Con-A used to label human red blood cells are shown. a) Kidney derived cells, b) Lung derived cells, c) Femoral bone marrow derived cells, d) Skin derived cells, e) Liver derived cells.

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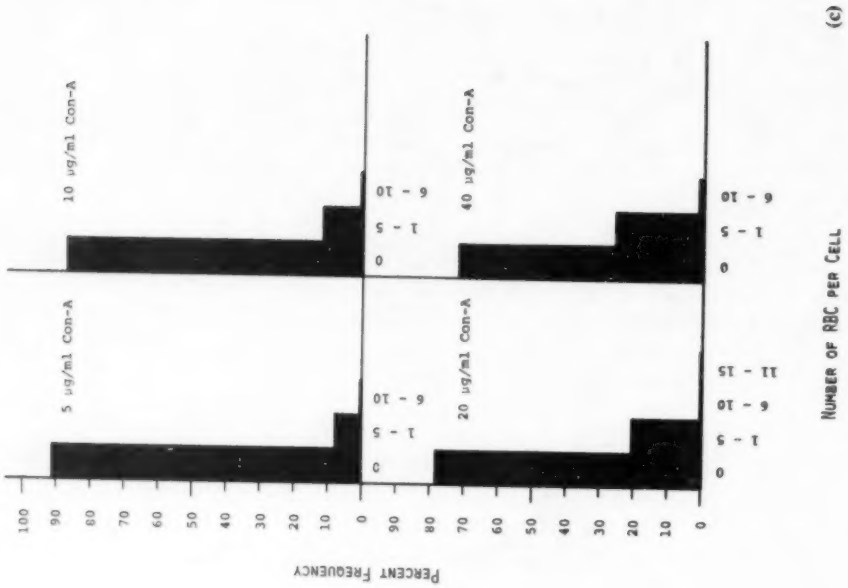
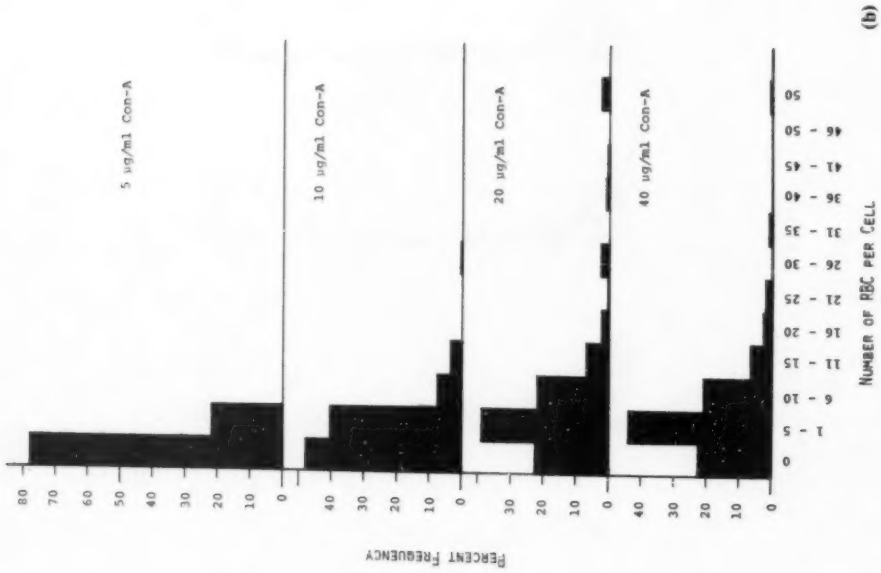
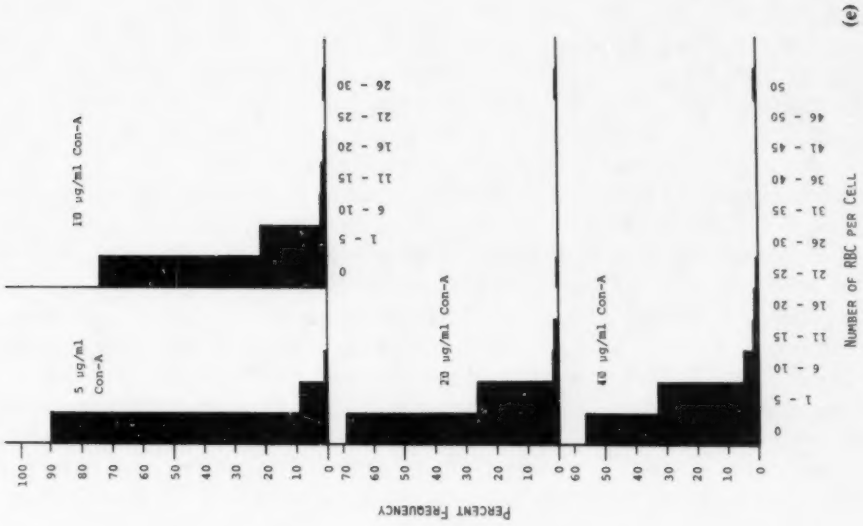
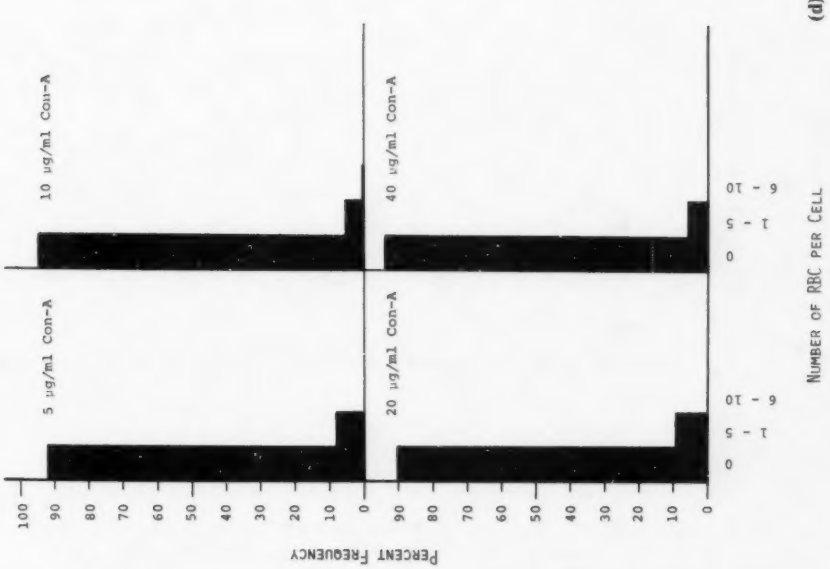

(c)
5 µg/ml Con-A
10 µg/ml Con-A
20 µg/ml Con-A
40 µg/ml Con-A
0
1 - 5
6 - 10
11 - 15
PERCENT FREQUENCY
NUMBER OF RBC PER CELL


(b)
5 µg/ml Con-A
10 µg/ml Con-A
20 µg/ml Con-A
40 µg/ml Con-A
0
1 - 5
6 - 10
11 - 15
16 - 20
21 - 25
26 - 30
31 - 35
36 - 40
41 - 45
46 - 50
50
PERCENT FREQUENCY
NUMBER OF RBC PER CELL


(e)
5 µg/ml Con-A
10 µg/ml Con-A
20 µg/ml Con-A
40 µg/ml Con-A
0
1 - 5
6 - 10
11 - 15
16 - 20
21 - 25
26 - 30
31 - 35
36 - 40
41 - 45
46 - 50
50
NUMBER OF RBC PER CELL
PERCENT FREQUENCY


(d)
5 µg/ml Con-A
10 µg/ml Con-A
20 µg/ml Con-A
40 µg/ml Con-A
0
1 - 5
6 - 10
NUMBER OF RBC PER CELL
PERCENT FREQUENCY

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Cells derived from the femoral bone-marrow, skin and liver showed almost no C-RBC adsorption ability, while lung cells showed weak C-RBC adsorption ability. A slight concentration dependency was observed up to a Con-A concentration of 20 μg/ml. The above four cell types showed a morphology quite similar with that of fibroblasts. Kidney-derived cells showed epithelial morphology and remarkably high C-RBC adsorption ability. Con-A unlabelled RBC were not adsorbed in the cells (data not shown). RBC labelled with Con-A at low concentrations (5 μg/ml) were easily adsorbed in kidney cells. However, even when the concentration of Con-A was increased to more than 10 μg/ml, adsorption distribution of C-RBC underwent little change. It is evident that cell populations having no C-RBC adsorption exist at a fixed ratio. As a result of the data shown in Figure 2 (c–e), cells adsorbing six or more C-RBC were regarded as Con-A reactivity positive and cells adsorbing no RBC as Con-A reactivity negative.

The patterns of C-RBC adsorption of cells obtained from various organs of the individuals (Figure 1) mostly showed, with the exception of kidney cells, the trends observed in Figure 2. Figure 3 shows in summary the ability to adsorb RBC labelled with Con-A at a concentration of 40 μg/ml. A comparison was made of the percent frequency levels of Con-A positive cells (cells adsorbing six or more C-RBC per cell) and Con-A negative cells (cells adsorbing no C-RBC). On comparison by age of cell donor (those less than four indicated as **y** in the figure and those more than 44 indicated as **a** in the figure), there was

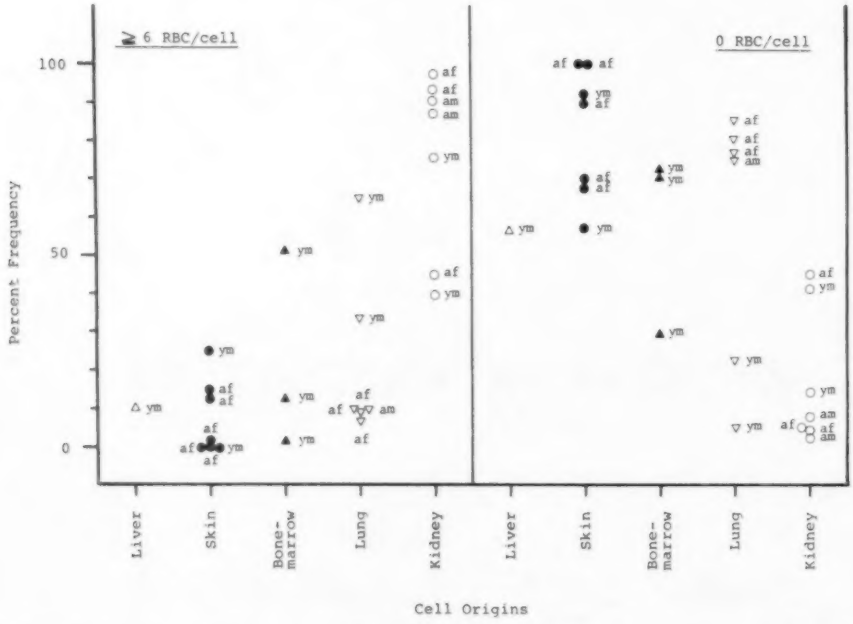

FIG. 3. C-RBC adsorption ability of culture cells established from a variety of normal human tissues. The left panel demonstrates the percent frequency of cells adsorbing six or more C-RBC per cell, and the right panel shows the percent frequency of cells adsorbing no C-RBC.
a: aged donor (donor age 44 years and over),
y: young donor (donor age four years and under),
m: male, **f:** female

no association between Con-A reactivity and donor age for skin and kidney fibroblasts. Lung fibroblasts showed a tendency for Con-A reactivity to be higher in younger cells than in aged cells. However, a large number of cases are needed in order to detect any cell type association between Con-A reactivity and donor age. Con-A reactivity of skin fibroblasts was very low. Con-A reactivity of liver and bone marrow cells was also low.

In Figure 4, the percent frequency of kidney cells adsorbing six or more C-RBC per cell is shown in relation to the concentration of Con-A. The cells were established from a portion of kidney tissues collected from a premature fetus (chromosome karyotype, 44 + XX), four-year-old boy, 62-year-old male, 70-year-old female, 79-year-old female, 80-year-old male, and 85-year-old male. C-RBC adsorption abilities for two cases of subjects four years and under, and those for five cases of 62 years and over were distributed rather widely. That is, there seems to be no correlation between the age of cell donors and Con-A reactivity.

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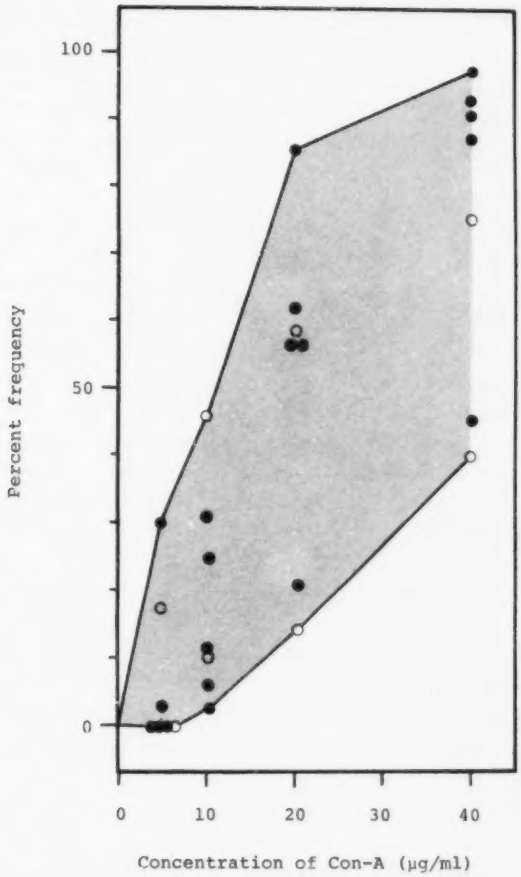

FIG. 4. Con-A concentration-dependency in C-RBC adsorption of kidney derived culture cells. The percent frequency of cells adsorbing six or more C-RBC per cell was obtained. There was no difference in C-RBC adsorption ability between tissue donors of four years and under (○) and those of 62 years and over (●).

DISCUSSION

Normal human diploid cells, which have a finite doubling potential in culture, are widely used as an *in vitro* aging model. Especially, fibroblasts established from lung (Ban *et al.*, 1980 a,b, 1981) and skin of human fetuses (Nikaido *et al.*, 1980) and skin tissue of human adults (Nikaido *et al.*, 1980) are frequently employed because they are easily available, can be established easily in culture, and have high proliferative ability. Cells derived from other organs are also excellent material for studies on aging (Ban *et al.*, 1982). Identification of species-specificity or tissue-specificity of the proliferating fibroblasts and other cells is very meaningful for the advancement of studies on cellular aging.

Furthermore, identifying the presence or absence of tumor-specificity in fibroblasts established from tumor tissues is of importance for promoting cancer studies.

Hayflick *et al.* (1961, 1965) suggested the presence of tissue-specificity in the *in vitro* life-span of fibroblasts. Oishi *et al.* (1981) reported that fibroblasts established from human prostate cancer show high reactivity to Con-A.

We studied Con-A reactivity of various cells for the purpose of identifying tissue-specificity of cells proliferating from normal human tissues. Materials were collected after they were confirmed to be free of either tumor or degradation. Cells proliferating from the kidney, lung, and skin are easily established, and proliferation of the established cells is good. Con-A reactivity of skin fibroblasts (Figures 2d and 3) is very low, and that of the liver cells (Figures 2e and 3) and femoral bone marrow cells (Figures 2c and 3) also seems to be low. Con-A reactivity of lung fibroblasts is slightly higher in the younger cells (Figures 2b and 3). However, kidney cells showed remarkably high C-RBC adsorption ability compared with the four types of cells mentioned above. Kidney cells are characterized by their Con-A reactivity at a low concentration (5 μg/ml) and the ratio of cells adsorbing 50 or more C-RBC per cell is high. However, many kidney cells do not adsorb C-RBC even when the concentration of Con-A used to label human red blood cells is increased (Figure 2). Some of the kidney cells that do not adsorb C-RBC demonstrate fibroblast-like morphology, but most are difficult to classify morphologically.

From the above observations, it is evident that Con-A reactivity is tissue-specific, and especially, that a large number of Con-A receptors exist on the membrane surface of kidney cells. However, Con-A reactivity of the individual kidney cells differed greatly, which suggests that kidney cells represent a mixed population of proliferating cells. However, an overwhelmingly large number of these cells have many Con-A receptors on their membrane surface. The number of Con-A receptors may possibly change during the cell cycle, but Aizawa *et al.* (1980, b) do not believe this to be true.

Most of the kidney cells had an epithelial morphology, which remained stable throughout the *in vitro* life-span of the cells. With some exceptions, it is very difficult to morphologically differentiate culture into epithelial cells and fibroblasts. Particularly, morphological kinetic changes, frequently observed as subcultures, are repeated after the first generation of culture cells is established. The Con-A hemadsorption test is useful as an indicator of changes in the growth kinetics of culture cells.

SUMMARY

An examination was made of the ability of culture cells that were established from various normal human tissues to adsorb concanavalin-A treated red blood cells (Con-A hemadsorption test). As most tumor cells have a high reactivity to Con-A, the Con-A

hemadsorption test is often used as a convenient indicator for identifying the neoplastic characteristic of fibroblasts. This study revealed, however, that cultured cells from normal kidney tissue have a remarkably high reactivity to Con-A. The morphology of the kidney cells was similar to that of epitheloid cells. However, Con-A reactivity of fibroblasts (or fibroblast-like cells) derived from lung, skin, bone-marrow, and liver was low. The Con-A hemadsorption test is not only useful for identification of neoplastic cells, but also provides information of cellular specificity.

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BRIEF ANNOTATION

THE SIGNIFICANCE OF FACIAL SKIN TEMPERATURE DIFFERENCES IN AN ELDERLY FEMALE CHRONIC MENTAL HOSPITAL POPULATION

C. McDonald, M.D., M.R.C. Psych. and A.J.J. Perez-Gil, L.M.S., M.R.C. Psych.

(*Received* 25 *April* 1983)

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Benian (1975) has suggested that low nose tip temperature in old people is a sign of poor prognosis. Howell (1980) has recorded nose tip and circumambient temperatures in 54 men and 53 women. He found no correlation between nose tip temperatures and age but suggested a tendency toward bimodal distribution with age in both men and women. Howell did not relate nose tip temperatures to prognosis although he pointed out that certain patients show a falling blood pressure during the terminal stages of their illness and that this would be likely to lead to low nose tip temperature.

This study investigated nose tip temperatures in 57 female in-patients in the long-stay wards of a mental hospital. Using a SMEC-10 Electronic Thermometer manufactured by the Shuwa Co. Ltd., temperature readings were taken of nose tip, right cheek, left cheek and circumambience. These measures were repeated at four weekly intervals for three months. Correlations were sought between age and the differences between nose tip and either cheek and between age and the difference between nose tip and circumambience. None were found. The cases were grouped by diagnosis. Functional illnesses were grouped together and dementing patients were examined as a group, by dividing them into senile dements and senescents (McDonald 1969) and by dividing them into under 85 year olds and over 85 year olds. Means and standard deviations for the groups were calculated allowing the calculation of t tests for differences between the means. No significant intergroup differences were found on any temperature measure.

Finally those cases who had died within six months of the start of the study and those who died two years later were treated statistically in the same way. Again no differences were found in the temperature measures between these groups and any of the other diagnostic groups.

It is concluded that in this female mental hospital elderly population, nose tip temperature held no prognostic or diagnostic significance.

Requests for reprints to Dr. C. McDonald, Warlingham Park Hospital, Warlingham, Surrey, England.

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THE EFFECT OF AGING ON VASOPRESSIN SYSTEM IN FISHER 344 RATS

VLASTA K. ZBUZEK, VRATISLAV ZBUZEK and WEN-HSIEN WU

Department of Anesthesiology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, 100 Bergen Street, Newark, New Jersey 07103

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Abstract—Vasopressin (VP) was measured by RIA in the plasma, neurohypophysis (NH) and hypothalamus (HT) of young (2 months), adult (12 months) and old (30 months) male Fisher 344 rats, ten of each age.

Plasma VP concentration was significantly lower in old compared to adult and to young rats. VP content in the NH expressed per mg weight was similar in all three groups, while the hypothalamic VP content was decreased in the aged rats. This suggests that reduced synthesis and release and/or increased degradation of VP occurs in aged rats. These data are in an agreement with our previous results obtained with Sprague-Dawley rats indicating that these differences are not strain-related.

In a pilot experiment, we further studied VP release from isolated perifused NH of young and old rats. Two glands of each age were simultaneously perifused in individual microchambers with medium TC 199 and subsequently stimulated by Locke's medium containing 56 mM K^+. Both, the initial and the basal VP release from the NH of the old rats, as well as the response to high K^+, were about a half that of the young rats. Thus, a decreased VP release may contribute to the findings of lower plasma VP concentration in aged rats.

INTRODUCTION

THE ROLE of vasopressin (VP) in the aging process is not well understood. The scarcity of available data allows us only to speculate that the rate of VP synthesis and the rate of VP release or both may be impaired in aging. The concentrations of neurotransmitters decline in the aged brain (Samorajki 1977). VP is considered to be such a neurotransmitter (Barker 1976). Aging reduces catecholamine metabolism in the hypothalamus and other parts of the brain (Finch 1979) while VP accelerates it (Tanaka *et al.* 1977). Finally, VP improves both memory and learning which are impaired in aging humans (Legros *et al.* 1978; Weingartner *et al.* 1981).

Recently, we reported a lower amount of VP in the hypothalamus (HT) but not in the neurohypophysis (NH) of old (30 months) male Sprague-Dawley (SD) rats, compared to young (three months). An undetectable plasma VP concentration in old rats suggested impairment of VP release in aging (Zbuzek and Wu, 1982). The present study was under-

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taken to verify whether similar results can be obtained with male Fisher 344 rats, which are preferably used in aging studies, to demonstrate that the previous data were not strain-related. In addition, we employed perifused NH explants from the young and old Fisher 344 rats, to study VP release.

METHODS AND PROCEDURE

Male Fisher 344 rats of 2, 12 and 30 months of age were provided by the National Institute on Aging colony maintained by Charles-River Breeding Laboratories. All animals were kept for ten days under the same conditions: constant temperature of 22.5 ± 0.5°C, 12:12 hours light/dark cycle and a rat Purina Chow diet and tap water *ad libitum*.

VP content in plasma, HT and NH

Ten rats in each age group were decapitated, and trunk blood was collected into heparinized plastic caps. Blood was centrifuged at 3000 rpm for 15 min. Plasma was frozen, and VP extracted within ten days. The NH including *pars intermedia* and the HT tissue block (approximately two mm from the midline bilaterally including *chiasma opticum* and *corpora mamillaria*), were quickly removed, weighed and dried in acetone for VP extraction. Plasma VP was extracted with cold acetone followed by petroleum ether (Robertson *et al.* 1973) and kept frozen until radioimmunoassay (RIA). Dried HT and NH were homogenized with two ml of 0.25% acetic acid, boiled for two minutes, chilled on ice and centrifuged at 3000 rpm for 20 min at 4°C. The supernatant was acidified with a drop of glacial acetic acid to keep pH below 3.0 and kept at 4°C until RIA.

In addition, the health status of the experimental animals was evaluated by gross autopsy. Also the spleen was removed and weighed, and hematocrits were measured in all animals.

VP release from isolated NH

The microperifusion system for VP release from isolated NH was developed in our laboratory. It consists of four 0.2 ml Delrin microchambers mounted in a 37°C environmental cabinet. Neurohypophyses from two young (2 months) and two old (30 months) male Fisher 344 rats were removed within two to three minutes following decapitation and placed in the microchambers. The glands were individually perifused for 4½ hours with synthetic medium TC 199 with Hank's salt (GIBCO), equilibrated with 95% O_2/5% CO_2, pH 7.4 at 37°C. Flow rate of perifusion (100 μl/min) was controlled by a roller pump (Gilson, Minipuls 2), and the perifusate of each microchamber was collected at 4°C with a 4-channel fraction collector (Gilson, Racetrack) enclosed in a chromatography chamber (Revco, Puffer-Hubbard Uni-term). Fractions were collected at 20, 10, 5 and again 10 minute intervals during initial traumatic release. basal release, stimulation with high K^+ Locke's solution (Locke, 1900) (that is 56 mM K^+ concentration obtained by substituting equivalent amount of Na^+ with K^+), and after stimulation, respectively. VP concentration was measured by RIA in 50 μl aliquots of the perifusate.

VP RIA

A double-antibody RIA developed in our laboratory was employed (Zbuzek and Wu, 1982). Briefly, monoiodinated 8-arginine vasopressin-^{125}I (New England Nuclear, 500 μC/μg) as a labeled antigen and synthetic AVP (Bio-Ria, 350 IU/mg, Lot #B582), was employed for calibration curve. Unknown samples in duplicate, four replicates of each concentration of the calibration curve and blanks and eight references were incubated at 4°C for three days and one day after adding second antibody. Samples were centrifuged at 3000 rpm for 40 minutes, decanted using decanting device (Beckman) and radioactivity measured in a gamma counter (Beckman 4000) and expressed as bound/reference. VP concentration was computed as pg/sample (Beckman DP-5000).

Statistical analysis

VP release from the isolated NH was expressed for each gland as pg/min/mg wet tissue. All other results are presented as mean ± SE and analyzed by analysis of variance. Tukey test was used to calculate the significance between the groups.

RESULTS

Autopsy revealed testicular interstitial tumors in three out of ten old rats. Otherwise all organs in rats of all three groups were normal by visual examination. Since all measured parameters (plasma VP concentration, VP content in the NH and the HT, body and NH

TABLE I. COMPARISON OF THE BODY WEIGHT AND THE WEIGHT OF NEUROHYPOPHYSIS (NH) OF YOUNG, ADULT AND OLD RATS

	No of Rats	*Body Weight (g)*	*NH Weight (mg)*
YOUNG (Y) (2 months)	10	231.2 ± 3.3	1.08 ± 0.05
ADULT (A) (12 months)	10	383.8 ± 4.8	1.43 ± 0.06
OLD (O) (30 months)	10	337.1 ± 4.6	1.49 ± 0.07
SIGNIFICANCE (p value)	–		
Y : A	–	<0.001	<0.001
Y : O	–	<0.001	<0.001
A : O	–	N.S.	N.S.

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weights) were similar to those of the other animals within the group, these three were not excluded from the study. All animals exhibited normal hematocrits, and none had splenomegaly secondary to infectious disease. There were no significant differences between the adult and old animals in their body weight and the weight of the NH. (Table I).

VP content in plasma, HT and NH

Plasma VP concentration was highest in the young (15.4 ± 0.7 μU/ml), slightly lower in the adult (12.8 ± 1.0 μU/ml; N.S.) and lowest in the old (3.6 ± 0.4 μU/ml, <0.001 compared to adult or young) rats. (Figure 1).

VP content in the HT was also highest in the young (16.7 ± 3 mU/gland), slightly

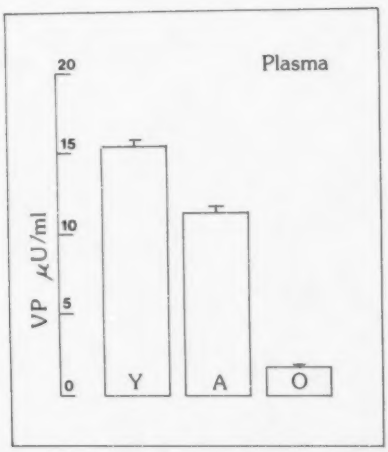

FIG. 1. Plasma vasopressin (VP) concentration in young (Y), adult (A) and old (O) male Fisher 344 rats. (1μU = 2.86 pg VP).

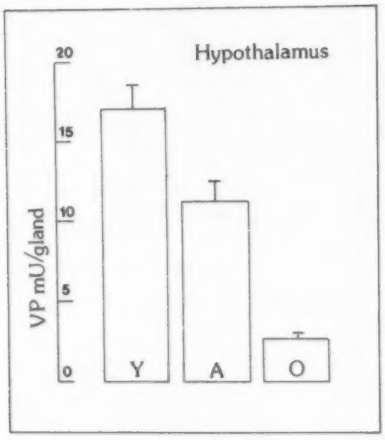

FIG. 2. Vasopressin (VP) content in the hypothalamus of young (Y), adult (A) and old (O) male Fisher 344 rats. (1 mU = 2.86 ng VP).

lower in the adult (11.3 ± 2.2 mU/gland, N.S.) and lowest in the old (2.6 ± 0.3 mU/gland; <0.01 compared to young; <0.05 compared to adult) rats. (Figure 2).

VP content of the NH, expressed per gland was the lowest in the young (564.9 ± 23.3 mU), highest in the adult (865.6 ± 67.6 mU; <0.01). In the old group it was higher (736.1 ± 53.8 mU; <0.05) compared to young, but not significantly different from the adult rats. However, when expressed per mg NH there were no significant age differences in the VP content of the NH of any age groups: 530.5 ± 29.1 mU in young; 609 ± 40.8 mU in adult; 506.3 ± 45.1 mU in old rats. (Figure 3).

VP release from the NH

The spontaneous VP release from perifused NH explant and the response to 20 minute stimulation by 56 mM K^+ are presented in Figures 4 and 5. The mean NH weight of young

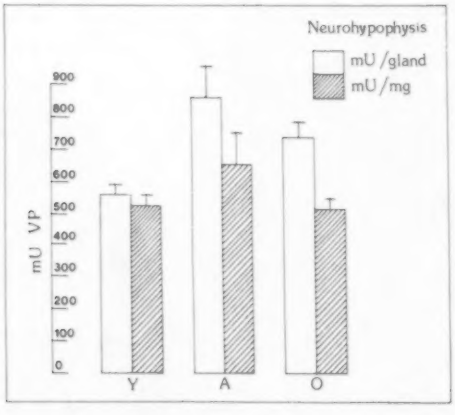

FIG. 3. Vasopressin (VP) content in the neurohypophysis of young (Y), adult (A) and old (O) male Fisher 344 rats, expressed per total gland (empty columns) and per mg wet tissue (shaded columns). (1 mU = 2.86 ng VP).

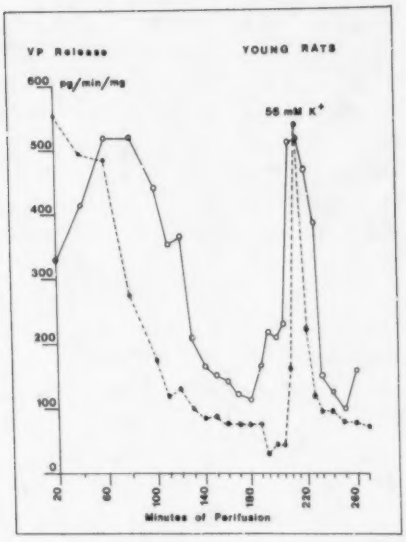

FIG. 4. Spontaneous vasopressin (VP) release from two isolated and individually perifused neurohypophysis of young (2 months) male Fisher 344 rats and their response to high K^+ Locke's medium. The VP release is expressed in pg/min/mg wet tissue. (1 pg = 0.35 μU VP).

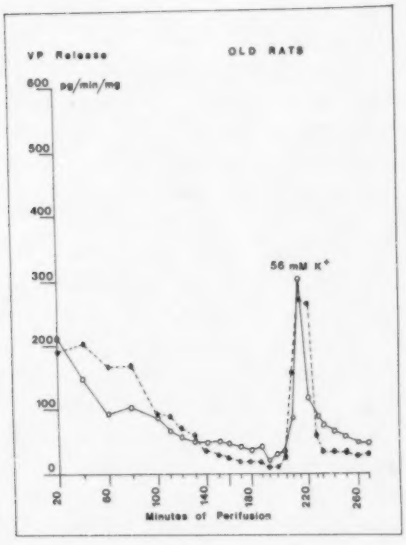

FIG. 5. Spontaneous VP release from two isolated and individually perifused neurohypophysis of old (30 months) male Fisher 344 rats and their response to high K^+ Locke's medium. The VP release is expressed in pg/min/mg wet tissue. (1 pg = 0.35 μU VP).

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rats was 0.92 mg (0.90 and 0.94 each) and 1.69 mg (1.84 and 1.54 each) in old rats respectively. The total VP release during first 100 minutes (traumatic release induced by the dissection of the gland) was 2073 pg/mg NH (1969 and 2177 each) from the young NH, while it was as low as 623 pg/mg NH (818 and 428 each) from the old NH. The average "basal" release from the young NH was 140 pg/min/mg NH (76 and 204 each) and 34 pg/min/mg NH (30 and 38 each) from the old NH. During the stimulation with 56 mM K^+ the total VP release was 1452 pg/20 min/mg NH from the young (1041 and 1863 each) and as low as 658 pg/20 min/mg NH (741 and 576 each) from the old NH.

DISCUSSION

Our results obtained in aged Fisher 344 rats indicate decreased plasma VP concentration, decreased VP content in the HT but not in the NH, compared to young rats. Hence, these data are similar to those obtained by us in Sprague-Dawley rats (Zbuzek and Wu, 1982). These results support our hypothesis that during aging, VP synthesis may be reduced while VP storage in the NH is not impaired. Furthermore, plasma VP concentration may reflect reduced VP release or increased degradation of VP. Similar age-dependent differences in the rate of synthesis and secretion of VP were postulated earlier by Turkington and Everitt (1976) who reported significant decreases in the urinary excretion and plasma concentrations of VP in aged Wistar rats.

Comparable data on reduced VP content in the HT of aged rats were reported by Lansfield *et al.* (1980) in Fisher 344 strain and by Dorsa and Bottemiller (1982) in Long-Evans strain. In addition, the latter authors also did not find any significant differences in the VP content between the old and young rats in the NH (VP/μg protein), indicating that the VP storage in the NH is not impaired by aging.

However, our data obtained on Fisher 344 and SD rats are in disagreement with the findings reported by Sladek *et al.* (1982) done on Fisher 344 rats. They reported decreased VP content in the NH (VP/mg tissue) in the old rats compared to the young animals. Also, their results indicate no difference in plasma VP concentration and in VP content in dissected supraoptic (SON) and paraventricular (PVN) nuclei, between young and old rats, contradicting our results. Sladek *et al.* suggested that the reduced VP content in the median eminence rather than in the SON and PVN may explain the total lower hypothalamic VP content found in the aged rats by others (Landfield *et al.*, 1980). Recently, Dorsa and Bottemiller (1982) reported VP content in different areas of the rat (Long-Evans) brain. Indeed, they found lower amount of VP in median eminence of old (26 months) compared to young (three months) rats. On the other hand, these authors found significantly lower VP content in SON and PVN and in other hypothalamic as well as extra hypothalamic nuclei, except a higher VP content in dorsal septum, in aged rats.

Considering well known strain-related differences between SD and Fisher 344 rats, the old SD rats are characterized by a greater body weight and a distinctly larger NH than the adult (12 months), compared to Fisher 344 rats of the identical ages. In our recent work (Zbuzek and Wu, 1982), we found the highest total VP content in the NH of old SD rats, lower in the adult and the lowest in the young rats, reflecting the glandular size. However, when VP was expressed per mg tissue, there was no difference among the ages. This is not the case in Fisher 344 rats. There is no difference in the weight of NH between 12 and 30 months old rats, as reported by Sladek *et al.* (1982) and confirmed by our experiments. The highest total VP content was found in the NH of the adult, lower in the old and the lowest in the young rats. Hence, it appears that in Fisher 344 rats the VP content in the

NH increases with the age, at least up to 12 months. This seems to be in agreement with the results obtained in Wistar rats by Cannata and Tremezzani (1976). They demonstrated by electron microscopy thicker axons of the neurosecretory cells and an increase in the number of granules per axon as a function of age. However, these authors did not study the changes in the NH beyond 12 months of age. Our results in Fisher 344 rats allow us to speculate that during the aging process, the total VP content in the NH of aged animals exhibits a declining tendency, because the old rats had lower VP content than the adult group, but still significantly higher compared to young group. Furthermore, some of the aged animals exhibited VP content in the NH within the range of the young group, perhaps reflecting a "faster" (lower VP content) and a "slower" (yet high VP content) individual aging process. Further studies in a larger population are required to answer this question.

A low plasma VP concentration in the aged Fisher 344 rats compared to young ones supports our previous data from SD rats (Zbuzek and Wu, 1982). It is also in agreement with the report of Turkington and Everitt (1976) on Wistar rats. Since our experiments were carried out in the summertime, the relatively high plasma VP concentration in our young (15.4 ± 0.7 μU/ml) and adult (12.8 ± 1.0 μU/ml) rats may reflect seasonal variations. High plasma VP concentration in the summer and low in the winter was reported earlier by us in young SD rats (Zbuzek and Wu, 1979).

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The data from microperifusion of individual NH showed a decreased spontaneous VP release from the gland of the old rats and also a reduced response to stimulation by high K^+ Locke's solution. This supports our hypothesis of a reduced VP release due to aging. Since only two glands of each age were perifused, the results cannot be analyzed statistically. However, this pilot experiment strongly indicates that the VP release from the old NH is about half that of the young ones. Thus it suggests that the reduced VP release may contribute to a decreased plasma VP concentration in old rats.

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AGE-DEPENDENT CHANGES IN RAT LIVER MICROSOMAL NADPH CYTOCHROME C (P-450) REDUCTASE: A KINETIC ANALYSIS

DOUGLAS L. SCHMUCKER and ROSE K. WANG

Cell Biology & Aging Section, Veterans Administration Medical Center, the Department of Anatomy and the Liver Center, University of California, San Francisco

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Abstract—Rat liver microsomal NADPH cytochrome c (P-450) reductase (EC 1.6.2.4) exhibits several marked age-dependent changes, including a decline in specific activity, reduced inducibility and enhanced thermostability, which are indicative of an alteration in the quality of this enzyme. The present study examined the kinetic profile of the microsomal-bound enzyme in an effort to further define the effects of aging on the hepatic mixed function oxidase system. Intact microsomes isolated from young adult (3 months), mature (16 months) and senescent (27 months) rats were subjected to an extensive double reciprocal kinetic analysis employing NADPH and cytochrome c as substrates. The K_m values obtained with the initial substrate (NADPH) remained unchanged with animal age, whereas there was a decline in this parameter for the artificial acceptor substrate, cytochrome c. The V_{max} values for both substrates were reduced as a function of increasing age, perhaps reflecting a concomitant decline in the relative amount(s) of efficient reductase in the microsomes.

INTRODUCTION

A NUMBER of clinical studies have established that aging causes a significant decline in the disposition rates of numerous drugs (see Schmucker, 1979 for a review). Although a variety of factors may contribute to this decline, including diminished splanchnic blood flow and reduced renal clearance of metabolites, there is considerable evidence which suggests that aging also results in a decrease in the hepatic capacity to metabolize drugs (Liddell, Williams and Briant, 1975; Jori, DiSalle and Quadri, 1972; Hayes, Langman and Laursen, 1975; Triggs *et al.*, 1975; Houghton, Richens and Leighton, 1975). Such clinical findings have been corroborated by both *in vivo* and *in vitro* experimental studies which demonstrated marked age-related declines in the activities of liver drug-metabolizing enzymes in rats (Schmucker and Wang, 1980, 1981; Rikans and Notley, 1982; Jayaraj and Richardson, 1981; McMartin *et al.*, 1980). Most drugs or xenobiotics which undergo hepatic biotransformation prior to their elimination are metabolized by the liver's microsomal mixed function oxidase system (MFOS).

Please address all correspondence to Douglas L. Schmucker, Cell Biology & Aging Section (151E), Veterans Administration Medical Center, 4150 Clement Street, San Francisco, CA 94121, (415) 750-2101.

Our previous studies have shown that the non-induced activities of several MFOS enzyme components decline during aging and that the MFOS in senescent rats does not respond to phenobarbital as rapidly or as extensively as does that in the livers of young or mature animals (Schmucker and Wang, 1981). These data correlated well with stereological evidence of an age-related loss of hepatocyte smooth surfaced endoplasmic reticulum, the primary site of the MFOS, in rats between maturity and senescence (Schmucker, Mooney and Jones, 1977; 1978). Still, these studies did not resolve the nature of the age-related changes in the MFOS, for example, was there less enzyme(s) or merely enzyme(s) with reduced efficiency in old rats?

The recent isolation, purification and characterization of an important MFOS enzyme —NADPH cytochrome c (P-450) reductase [E.C. 1.6.2.4]—from the livers of rats of various ages has provided additional insight into this question. The activity of the purified enzyme exhibits a two-fold decline between 3 and 27 months of age and this has been attributed to a 40–45% decline in the enzyme activity/immunoprecipitable enzyme ratio during the same age span (Schmucker and Wang, 1983). These data clearly indicate a change in the quality of the reductase isolated from old rats versus younger animals. The primary objective of the present study is to determine if this "altered" enzyme also exhibits different kinetic characteristics. In order to achieve this goal, microsome-bound NADPH cytochrome c (P-450) reductase from young adult, mature and senescent rats was subjected to an extensive bisubstrate kinetic analysis.

MATERIALS AND METHODS

Male Fischer 344 rats (CDF) at 3 (young adult), 16 (mature) and 27 (senescent) months of age and corresponding to 100%, 100% and < 50% survivorship levels, respectively, were obtained from the NIA barrier aging colony and used as the source of hepatic microsomes. Upon arrival the animals were housed in barrier cages suspended over kiln-dried hardwood shavings to minimize the possibility of inducing hepatic monooxygenases by volatile exogenous compounds (Gold and Widnell, 1975). The rats were fed laboratory chow and water *ad libitum*, but were subjected to a 24 hour fast prior to use in order to reduce the amount of hepatic glycogen.

A laparotomy was performed under sodium pentabarbital anesthesia, the liver was perfused *in situ* with cold, oxygenated saline, removed and microsomes prepared by a routine procedure (Anthony, 1973). The washed microsomes were relatively free of contamination by other identifiable cell organelles as revealed by electron microscopic examination (Schmucker and Wang, 1981). Microsome-bound, rather than solubilized and purified, enzyme was chosen for these studies for several reasons, including: 1) the microsomal milieu, that is the phospholipid constituents, more correctly reflects the *in vivo* environment of the enzyme in comparison to a reconstituted system and 2) the expense of mature and senescent animals prohibited the preparation of sufficient amounts of purified reductase to permit the use of reconstituted systems. The microsomes were suspended in 0.25 M sucrose in 0.05 M potassium phosphate buffer (pH 7.4; ~20 mg • ml^{-1}) and stored at −70°C until used.

NADPH cytochrome c (P-450) reductase activity was estimated by measuring the rate of cytochrome c reduction using an extinction coefficient of 21 cm^{-1} at 550 nm (Masters, Williams and Kamin, 1967). All analyses were conducted at pH 7.7 and 37°C using a Gilford recording spectrophotometer equipped with a thermal block. Bisubstrate kinetic analyses were performed using cytochrome c as the acceptor substrate at concentrations ranging from 2 to 35 μmoles. The use of higher concentrations of this substrate were not possible since the absorbance attributed to cytochrome c alone interfered with the assay. The concentration of the primary substrate, NADPH, was varied between 9 and 900 μmoles. Masters and co-workers reported that the routine assay system represented the maximum initial reaction velocity obtainable at infinite concentrations of both substrates (Masters *et al.*, 1965). These substrate concentrations were selected on the basis of published values employed in reconstituted systems using purified enzyme. Each assay system contained 40 μgm of microsomal protein. Each point on the double reciprocal plots (Lineweaver-Burke) represents the average of at least two analyses. The entire bisubstrate kinetic analysis was conducted on two separate occasions with similar results. The protein content was measured by the method of Lowry *et al.* using bovine serum albumin as the standard (Lowry *et al.*, 1951). The inhibition studies were performed under conditions similar to those described by Segel (1975).

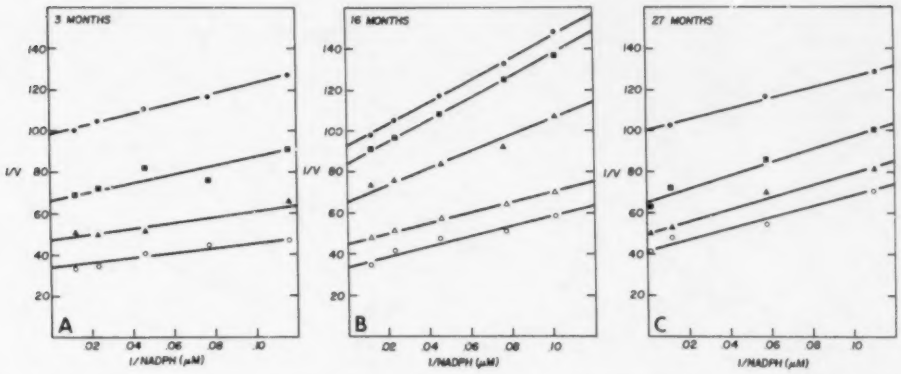

FIG. 1. Primary double reciprocal plots of rates of reaction of hepatic microsome-bound NADPH cytochrome c (P-450) reductase from young adult (3 months) [A], mature (16 months) [B] and senescent (27 months) [C] rats as a function of NADPH concentration at different fixed concentrations of cytochrome c. The concentration of the acceptor substrate (cytochrome c) was maintained between 1.7 and 26 μmoles for variable concentrations of the primary substrate (NADPH). Extensions of the plots beyond the terminal data points represent extrapolations and are intended to facilitate the estimation of apparent V_{max} values. The initial rates of reaction are expressed as Δ Absorbance Units • min^{-1}.

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RESULTS

The specific activity of microsome-bound NADPH cytochrome c (P-450) reductase used in the present studies exhibited an age-related decline, that is 0.26 to 0.16 μmoles • min^{-1}mg $protein^{-1}$ [40%] between young adult and old rats. Bisubstrate kinetic analyses of the rate of cytochrome c reduction versus the concentration of NADPH at fixed concentrations of cytochrome c yielded essentially parallel Lineweaver-Burke plots regardless of animal age (Figure 1). Non-intersecting plots were also obtained when the acceptor, cytochrome c, was the variable substrate, although the slopes and, thus, the apparent K_m values were somewhat different as a function of animal age (Figure 2). The

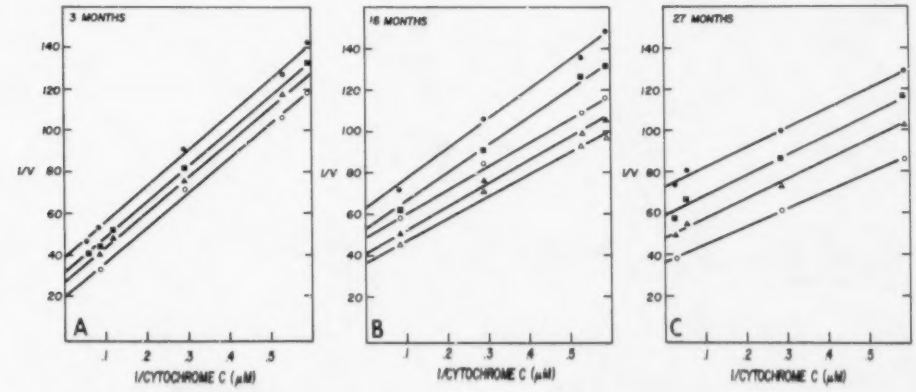

FIG. 2. Primary double reciprocal plots of rates of reaction of rat liver NADPH cytochrome c (P-450) reductase from young adult (3 months) [A], mature (16 months) [B] and senescent (27 months) [C] animals *vs* variable concentrations of cytochrome c at several different fixed concentrations of NADPH [9 to 90 μmoles]. The extensions of the slopes beyond the terminal data points represent extrapolations to permit the determination of the apparent V_{max} values. The initial reaction rates are expressed as in Fig. 1.

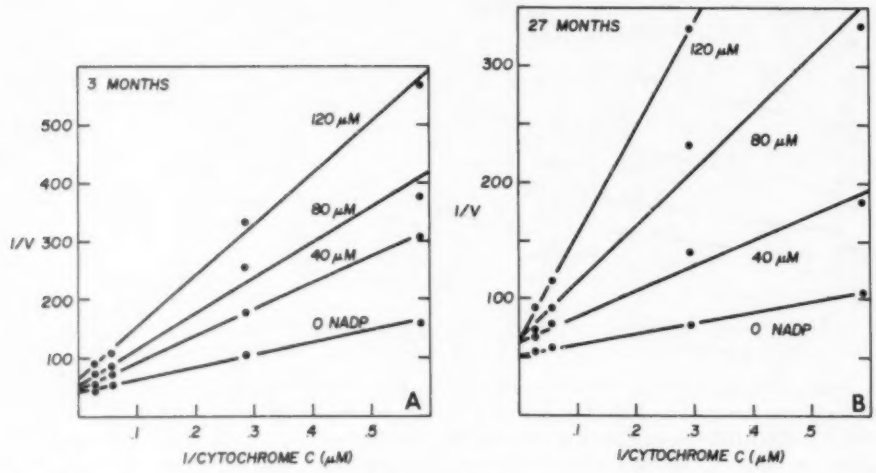

FIG. 3. Inhibition of rat liver NADPH cytochrome c (P-450) reductase as a function of the concentration of NADP in microsomes from young adult (3 months) [A] and senescent (27 months) [B] animals. The initial rates of reaction were determined at 1 μmole NADPH *vs* a variable concentration of cytochrome c in the presence of 0, 40, 80 and 120 μmoles of NADP. Extensions of the plots beyond the terminal data points represent extrapolations. The reaction rates are expressed as in Fig. 1.

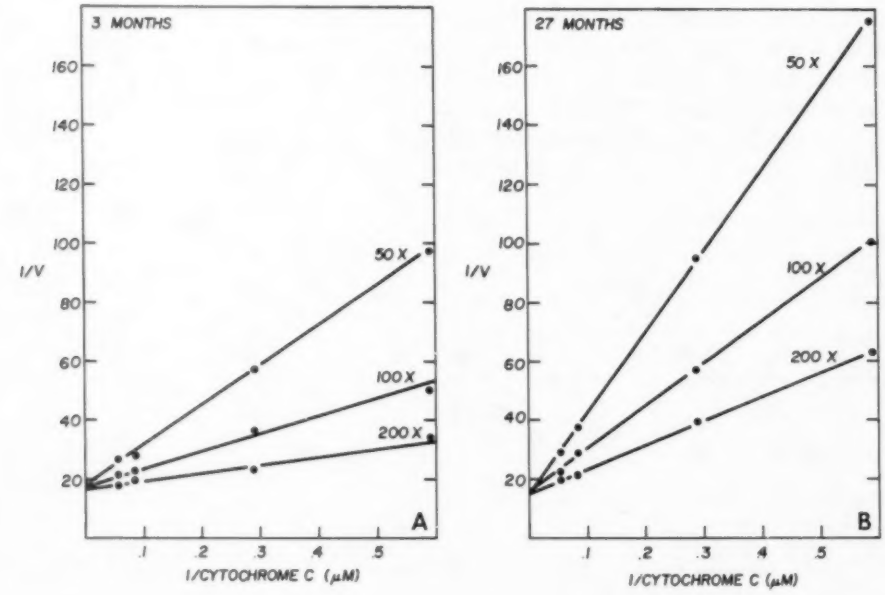

FIG. 4. Lineweaver-Burke plots of the rates of reaction for hepatic NADPH cytochrome c (P-450) reductase from young adults (3 months) [A] and senescent (27 months) [B] rats determined with the concentrations of NADPH and cytochrome c varied at a constant ratio (50, 100 and 200 X). The concentrations of NADPH were varied according to a fixed ratio expressed by [NADPH] = X • [Cytochrome c]. Extensions of the slopes beyond the terminal data points represent extrapolations. The initial reaction rates are expressed as in Fig. 1.

highest concentrations of cytochrome c resulted in substrate inhibition and, as a result, some of the data points were omitted from these double reciprocal plots.

Product inhibition analyses using NADP are demonstrated in Figure 3. A competitive-type inhibition resulted with increasing concentrations of NADP versus cytochrome c using enzyme from young adult and senescent animals. Subsequent replots of the apparent K_m's versus inhibitor concentration (NADP) yielded K_i's of approximately 59 and 15 μmoles for enzyme from 3 and 27 months-old animals, respectively. Varying the substrate concentrations, NADPH and cytochrome c, at different fixed ratios, that is NADPH/cytochrome c ratios of 50, 100 and 200, yielded linear intersecting plots as anticipated for the suspected enzyme mechanism (Figure 4). No effort was made to measure inhibition in response to increasing concentrations of reduced cytochrome c since the absorbance of this substrate alone interfered with the reaction assay.

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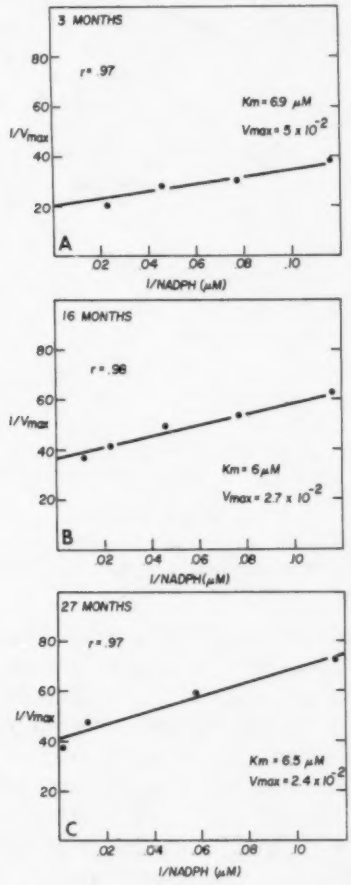

FIG. 5. Secondary double reciprocal plots of hepatic microsome-bound NADPH cytochrome c (P-450) reductase from young adult (3 months) [A], mature (16 months) [B] and senescent (27 months) [C] rats ($Vmax_{app}^{-1}$ *vs* S^{-1}). The rates of reaction were determined at a variable concentration of NADPH. The slopes were extended beyond the terminal data points to S^{-1} to facilitate the determination of K_m^{-1}. The initial rates of reaction are expressed as in Fig. 1. r = correlation coefficient.

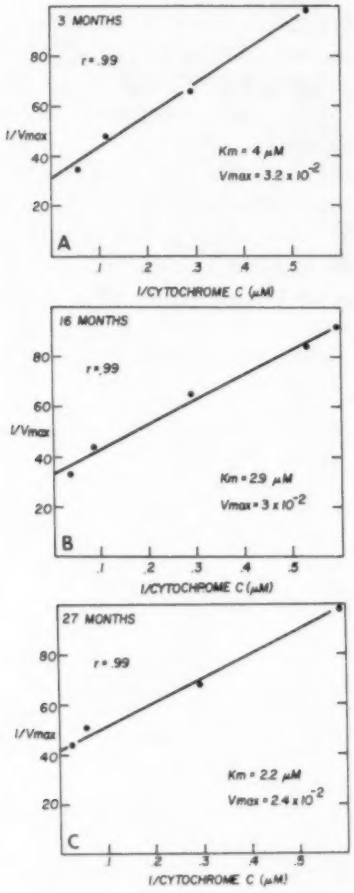

FIG. 6. Secondary Lineweaver-Burke plots similar to those in Fig. 5 except determined at variable concentrations of cytochrome c. As in Fig. 5 the slopes were extrapolated beyond the terminal data points to determine the K_m^{-1} values. The initial rates of reaction are expressed as in Fig. 1. r = correlation coefficient.

The true K_m values for mechanisms exhibiting parallel Lineweaver-Burke plots can be estimated using replots of the apparent V_{max}'s versus substrate concentration. The secondary double reciprocal plots ($Vmax_{app}^{-1}$ versus S^{-1}) for both substrates are shown in Figures 5 and 6. The plots for NADPH as the variable substrate yielded essentially similar K_m values of 6.9, 6 and 6.5 μm for microsome-bound enzyme isolated from young adult, mature and old animals, respectively. However, the corresponding V_{max} values exhibited an estimated 50% decline, that is $5 \cdot 10^{-2}$ to $2.4 \cdot 10^{-2}$. Secondary Lineweaver-Burke plots for the remaining substrate, cytochrome c, exhibited declines in K_m (~45%) and V_{max} (~25%) between young adults and senescent rats.

DISCUSSION

We are aware that the use of unpurified enzyme, that is microsome-bound, for kinetic analysis presents considerable difficulties with regard to the interpretation of the resultant

data. However, the reasons cited above, especially the exhorbitant cost of mature and truly senescent rats, necessitated our use of intact microsomes, that is membrane-bound NADPH cytochrome c (P-450) reductase, in the present study. Regardless, these are the first reported kinetic data on this very important MFOS enzyme, either purified or microsome-bound, as a function of animal age.

The parallel lines obtained in the primary Lineweaver-Burke plots using either substrate are indicative of a "ping-pong" mechanism (Cleland, 1963). Essentially, this kinetic profile suggests that the initial substrate (NADPH) interacts with the reductase to yield a product (NADP) and a modified enzyme which subsequently reacts with the second substrate or acceptor, cytochrome c (or one of the cytochromes P-450 $_{LM}$). Using solubilized and purified rat and pig liver NADPH cytochrome c (P-450) reductase, Masters and coworkers reported similar parallel Lineweaver-Burke plots (Masters *et al.*, 1965). However, there has been considerable controversy surrounding the interpretation of these data and the true mechanism of this particular enzyme. Phillips and Langdon (1962), as well as others (Dignam and Strobel, 1977), observed intersecting primary double reciprocal plots using purified reductase and concluded that the mechanism was sequential. The latter investigators attributed these conflicting data to Masters *et al.* using a low ionic strength buffer (50 mM). Phillips and Langdon (1962) demonstrated that increasing the ionic strength (~ 20-fold) resulted in increases in the $V_{max}^{cyto\ c}$ (3-fold) and the K_m^{NADPH} (25-fold), as well as in the K_i^{NADP}. Interestingly, Yasukochi and Masters (1976) used both 50 and 300 mM buffers and noted a two-fold increase in the specific activity of the microsome-bound enzyme in the latter buffer. The buffer employed in the enzyme assay system in the present study was similar to that of Masters *et al.* (1965), that is 50 mM. The lower rates of cytochrome c reduction that occur in low ionic strength buffer may not yield Lineweaver-Burke plots with obvious intersecting slopes and, thus, may be subject to misinterpretation.

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However, the linear and intersecting Lineweaver-Burke plots obtained when the substrates were varied at different fixed ratios are indicative of a "ping-pong" rather than a sequential mechanism (Segel, 1975). Furthermore, the product inhibition analyses demonstrated a competitive-type inhibition between NADPH and NADP, suggesting that both the substrate and the product bind to the same form of the enzyme—a finding consistent with a "ping-pong" mechanism. The product inhibition plots and the estimated K_i's for NADP suggest that NADPH cytochrome c (P-450) reductase from senescent rats is more sensitive to product inhibition in comparison to that from younger animals. For example, 80 μmoles of NADP resulted in ~50% inhibition of enzyme from old rats, whereas 120 μmoles of product were required to achieve a similar level of inhibition of microsome-bound reductase isolated from young animals. The K_i^{NADP} for membrane-bound reductase from young rats is approximately 30-fold higher than that reported for purified enzyme (Williams and Kamin, 1962; Masters *et al.*, 1965). This apparent discrepancy may reflect considerable binding of the product (NADP) by NADPH-dependent enzymes other than NADPH cytochrome c (P-450) reductase in the intact microsomes. Thus, greater concentrations of product would be required to achieve equivalent levels of inhibition.

Secondary Lineweaver-Burke plots ($Vmax_{app}^{-1}$ versus S^{-1}) yield slopes which intersect the S^{-1} intercept at K_m^{-1} for a given substrate. The absence of any significant change in the K_m^{NADPH} for microsome-bound NADPH cytochrome c (P-450) reductase suggests that this enzyme-substrate affinity is unaffected by aging. In addition, the K_m^{NADPH} values obtained

(6, 6.9 and 6.5 μM) are in agreement with true or apparent K_m's observed in reconstituted systems using purified reductase from young animals and several different acceptor substrates (5–6 μM) (Williams and Kamin, 1962; Masters and Kamin, 1965; Dignam and Strobel, 1977). The age-associated decline in the V_{max} for this substrate may reflect the suspected age-related decrease in the membrane concentration of efficient enzyme as has been suggested by the marked reduction in the enzyme activity/immunoprecipitable enzyme ratio (Schmucker and Wang, 1983).

The age-related decline in the K_m for cytochrome c suggests that the enzyme in old rats exhibits a stronger affinity for this acceptor substrate than does reductase in young or mature animals. One possible explanation may reflect the concomitant loss of cytochrome P-450 (50%), the preferred *in vivo* acceptor substrate of this enzyme (Schmucker and Wang, 1980). Furthermore, Dignam and Strobel (1977) demonstrated that the K_m value for the artificial substrate (cytochrome c) was approximately 100-fold greater [6.7–10μM] than that for cytochrome P-450 (0.1–0.08 μM) in reconstituted systems. Since the intact microsomes were used in the present study and the membranes of old animals contain significantly less cytochrome P-450 than do those of young rats, the competition between cytochrome c and cytochrome P-450 for available electrons may be reduced in the former. Therefore, the larger cytochrome c/cytochrome P-450 ratio in the assay systems containing microsomes from old animals may account for the apparent decline in the K_m for the artificial substrate.

The decrease in V_{max} is probably a direct consequence of the age-related decline in the concentration of functional enzyme in the membranes of the senescent animals. However, our values are in agreement with those reported by Phillips and Langdon (1962) ($2.7 \cdot 10^{-2}$) using partially purified enzyme in a buffer system of identical ionic strength as that employed in the present study (50 mM). In essence, the age-related decline in the functional capacity of the rat liver MFOS is due, in part, to qualitative and quantitative changes in several important components, including a loss of the important hemoprotein cytochrome P-450 and the accumulation of "altered" NADPH cytochrome c (P-450) reductase. The latter change is not reflected in marked differences in the basic kinetic properties of this enzyme.

In view of these data, we suggest that the pool of reductase in old rats is composed of two populations, a fully efficient enzyme and a relatively non-functional, yet immunoprecipitable protein or "altered" enzyme. In the event that the reductase pool in old animals consisted of enzyme exhibiting a complete spectrum of efficiencies, such age-related differences would most likely be expressed in greater changes in the kinetic profile.

SUMMARY

The objective of the present study was to determine whether the "altered" rat liver microsome-bound NADPH cytochrome c (P-450) reductase which accumulates in senescent animals exhibited a different kinetic profile than enzyme found in younger rats. Enzyme in young animals operates via a "ping-pong" mechanism and this particular feature is unaffected by aging. Furthermore, the K_m values obtained for both substrates, NADPH and cytochrome c, in all three age groups did not reflect the marked age-related changes in several other enzyme parameters, including the specific activity of the purified enzyme and the enzyme activity/immunoprecipitable enzyme ratio. Product inhibition analyses using NADP suggested that microsome-bound reductase from old rats is more sensitive to

competitive inhibition than enzyme obtained from younger animals. However, the relatively small age-related differences in the kinetic profile may be attributable to changes in several quantitative parameters, such as declines in the amounts of functional enzyme and cytochrome P-450.

Acknowledgements—The authors are indebted to Dr. Albert L. Jones for the use of his facilities and for support during the course of these studies. This research was supported by NIH grants AG00357 and AM25878, Liver Core Center grant (NIAMDD) P50 AM18520 and the Veterans Administration.

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The prize will be worth 20.000 Swiss Francs, and will be sponsored by SANDOZ LTD, Basel/Switzerland with the purpose of encouraging research in all areas of gerontology and geriatric medicine including biological, medical, psychological, social and other relevant aspects with special emphasis on multidisciplinary research programs.

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NO MALE-FEMALE DIFFERENCE IN *IN VITRO* LIFESPAN OF SKIN FIBROBLASTS FROM HUMANS AND MICE

P. EBBESEN

The Institute of Cancer Research (sponsored by the Danish Cancer Society), Radiumstationen, DK-8000 Aarhus C, Denmark

(*Received* 12 *May* 1983)

Abstract—To determine if the difference in mean survival time of males and females is related to the mechanism restricting the number of *in vitro* cell doublings, we established cultures of human skin fibroblasts and murine embryo cells. The maximum number of *in vitro* doublings was found to be very similar for the two sexes in both species. Furthermore, human cultures receiving repeated treatments with three alkylating carcinogens had the same *in vivo* survival as untreated controls.

INTRODUCTION

IN MANY mammalian species, including man and mice, females have a longer mean survival than males (Russell, 1966; Goodnick, 1975). This difference is often assumed to derive ultimately from influence exerted by the sex chromosomes. Interspecies differences in maximum survival time parallel interspecies differences in *in vitro* doubling potential of fibroblasts (Hayflick, 1977), suggesting a general aging mechanism which is also operative under *in vitro* conditions. At present, we are studying the possibility that intersex differences also are reflected in the *in vitro* doubling potential.

MATERIALS AND METHOD

Punch biopsies were obtained from 26 healthy adult *human* males aged 17–56 (mean 33) and 22 females aged 17–59 (mean 33). The uppermost area of dermis was dissected out (Harper, 1979) and seeded. We used Eagle's medium with Hank's buffer and 10% non-inactivated fetal calf serum of a batch containing 12,000 picogram of estron sulfate, 41 picogram of estron, less than 10 picogram of estradiol and 160 picogram of testosterone. Dihydrotestosterone, Δ-4 androstendion and dehydroepiandrosterone sulfate were not detectable[1]. From each primary culture four sublines were established in 75 cm^2 Falcon bottles and kept in different incubators; one subline from each donor was carcinogen treated. Bottles were harvested at near confluency, the cells counted, and 10^5 cells reseeded. Plating efficiency (percent attached 24 hours after seeding) and cloning efficiency (160 cells distributed to 200 Falcon microtiter wells) were determined every tenth passage. *Mouse* cultures were established by seeding 10^3 cells from 19-day-old sex-determined embryos. All *in vitro* doublings of cells from a given embryo took place in one 250 cm^2 bottle.

Alkylating chemical carcinogens (KaKunaga, 1977) *N-methyl-N'-nitro-N-nitrosoguanidine* (4N), *methyl methanesulfonate* (MS), and *4-nitro-quinoline-1-oxide* (NQO) were purchased from Merck, Darmstadt, West Germany. The human cultures to be treated were given medium without serum containing 10^{-6}, 10^{-5}, and $10^{-6.5}$

[1]The testing was kindly performed by Paul Bennet, The State Serum Institute, Copenhagen.

M, respectively, of carcinogen for one week. Each culture received 4N during the second passage, MS during the fifth passage and NQO during the eighth passage. Preliminary testing had shown the dose to be 1 log lower than the dose decreasing the number of cells. Statistics were carried out with Student's two-tailed t-test and Wilcoxon U-statistic.

RESULTS

Cultures were established from all *human* biopsies. Plating efficiency when reseeding was close to 65% in all tests and cloning efficiency very low (<1 percent). Obtained doublings were calculated on the assumption that all attached cells also divide. The mean doubling value was 43 ± 16 for the untreated cells from male donors and 44 ± 14 for the untreated cells from female donors. Carcinogen-treated cells from male donors reach a mean doubling value of 46 ± 14 while it was 48 ± 17 for the female donors. Neither t-test nor Wilcoxon's statistic showed any difference between the mean values at the 5% level.

Mouse cells had a primary plating efficiency of about 11% and a cloning efficiency of 3%. Eleven BALB/c male and 14 female fetus reached 9 ± 4 and 10 ± 4 doublings respectively while the figures for 22 C57/Bl males and 10 females were 11 ± 3 and 10 ± 5 doublings.

DISCUSSION

For humans the difference in mean survival time between males and females in the industrialized countries is of the order of five to seven percent. In laboratory mice the sex difference in survival time varies from twenty-five to zero percent depending on the way the animals are grouped in their boxes. When a box contains more than one male the mean survival time of the males will be lower than that of females irrespective of whether the males engage in physical fighting or not. However, when males are kept alone or in company with females, both sexes show a high mean survival time (Ebbesen, 1968). From the present work it appears that the maximum number of *in vitro* doublings of normal human and murine fibroblasts do not differ between the sexes to a degree detectable by the method used here. We, therefore, still favor the hypothesis that sex differences in survival time are largely dependent on interpersonal stress factors. That treatment with alkylating carcinogenic agents does not influence *in vitro* survival suggests that there may be no state intermediary between "normal" *in vitro* lifespan (Hayflick and Moorhead, 1961) and the immortalization so characteristic of malignant cells.

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A COMPARISON OF LIFE SPAN CHARACTERISTICS IN *DROSOPHILA*

EDGAR M. SCHNEBEL and JOSEPH GROSSFIELD

Department of Biology, The City College of the City University of New York, Convent Avenue and 138th Street New York, N.Y. 10031

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Abstract—A comparison of life span characteristics in 12 *Drosophila* species representing different degrees of phylogenetic relatedness and various ecological backgrounds was undertaken. Survival curve, LT50, LT100, range and mortality rate were compared at three different levels: intraspecific (male-female); interspecific (within species group triads); intergroup (among species group triads).

Data indicate that when differences are found within species groups, there is greater similarity between more closely related taxa. At all levels of comparisons, differences cannot be used to predict from one characteristic to another or from one sex to the other. The influence of both phylogenetic and ecological factors on patterns of results are evaluated, as well as the suggestion that at least two different mechanisms of aging may exist in the genus *Drosophila*.

The observed diversity in life span characteristics among *Drosophila* species groups and similarity within species groups, demonstrate the value of a comparative approach for studying aging.

INTRODUCTION

AN UNDERSTANDING of factors that influence longevity and the aging process is important in ultimately dissecting these phenomena at various levels of biological organization. The advantages of using insects in general, and *Drosophila* in particular, for such studies have been enumerated (Herman, *et al.*, 1971; Rockstein and Miquel, 1973; Lamb, 1979). Briefly, the genus *Drosophila* is large, has a worldwide distribution, and reflects an enormous ecological, physiological and behavioral diversity (Parsons, 1973; Throckmorton, 1975). These features make *Drosophila* an ideal organism for studies of lifespan and aging.

One method for gaining insights into the aging process is through the use of a comparative approach. Semispecies (not fully reproductively isolated), sibling species (morphologically identical but reproductively isolated), species groups and other related *Drosophila* taxa have been frequently used to study the similarities and differences of phenotypes at numerous levels of biological organization, including biochemistry, cytology, morphology, courtship behavior, and other behavioral and physiological responses (Spieth, 1952; Stone, *et al.*, 1960; Wasserman, 1960; Throckmorton, 1962; Brown, 1965; Ehrman, 1965; Hubby and Throckmorton, 1968; Richmond, 1972a,b; Ayala, *et al.*, 1974; Prakash, 1977; Ringo and Hodosh, 1978; Olivera, *et al.*, 1979; Parsons, 1980). These investigations have revealed patterns in which phenotypic similarities and differences correspond to phylogenetic similarities and differences. Studies of this

type have often been used to determine and/or confirm phylogenetic relationships. There are no data concerning phylogenetic comparative studies for life span characteristics in *Drosophila*. It is of value to determine whether degrees of genetic relatedness can be used not only as predictors of similarity in life span characteristics, but also as criteria for choosing species for subsequent analyses of aging mechanisms.

Comparisons of phenotypes have also been made in *Drosophila* using species from different environmental backgrounds. The rationale for this approach includes determination of limits in phenotypic response, the ecological and evolutionary significance of the response, and the mechanisms that account for differences in response (Levins, 1969; Grossfield, 1971; McKenzie and Parsons, 1974; Prince and Parsons, 1977; Richmond and Gerking, 1979; Cohet, *et al.*, 1980; Markow and Fogleman, 1981; Parsons and Spence, 1981). Again, however, ecological comparisons of species' life span characteristics are lacking and answers to the above questions remain unavailable.

Aging studies in *Drosophila* have primarily used only one species, *D. melanogaster*. As seen in Table 1, studies of *D. melanogaster* and a few other species have been carried out

TABLE 1. LONGEVITY IN WILD-TYPE STRAINS OF DROSOPHILA SPECIES

Species (Strain)	*Sex*	*Environmental Conditions: T°/RH%*	*Longevity (comments)**	*Reference*
D. melanogaster				
(Old Falmouth)	♂ ♀	25°/not stated	LT50:43; LT100:97 LT 50:40; LT100:95 (estimated)	Pearl and Parker, 1921.
(Old Falmouth)	sexes comb.	25°/not stated	LT50:39.5; LT100:77.5 (estimated)	Gonzalez, 1923.
(Swedish-R)	♂ ♀	25 ± 0.5°/not stated	LT50: 44 ± 3.9 LT50:51 ± 2.7	Baxter and Blair, 1967.
(Oregon-R)	♂	22 ± 1°/not stated	LT50:73; LT100:90 (estimated)	Atlan, *et al.*, 1969.
(Canton-S)	♂ ♀	not stated	$\bar{x}$ life span:50.4–52.9 $\bar{x}$ life span:41.9–59	Hall, 1969.
(Canton-S)	♀	25 ± 0.5°/50%	LT50: 79.4	Trout and Kaplan, 1970.
(Oregon-R)	♂	21 ± 1°/45%	LT50:95; LT100:114 (estimated)	Herman, *et al.*, 1971.
(Oregon-R)	♂	22 ± 0.5°/45%	LT50:92; LT100:119 (Estimated)	Miquel, 1971.
(Oregon-R)	not stated	25 ± 0.5°/50%	$\bar{x}$ life span:45 ± 2	Samis, *et al.*, 1971.
(Oregon-R)	not stated	22 ± 0.5°/45%	LT50:95; LT100:120 (estimated)	Miquel, *et al.*, 1972.
(Oregon-R)	not stated	25 ± 1°/50%	$\bar{x}$ life span:62 ± 3.5 maximum:110–120	Webb and Tribe, 1974.
(Oregon-R)	♂	23 ± 0.5°/not stated	$\bar{x}$ life span:78.1 maximum:98	Miquel, *et al.*, 1975.

(*continued*)

TABLE 1. (*con't*)

Species (Strain)	*Sex*	*Environmental Conditions: T°/RH%*	*Longevity (comments)**	*Reference*
D. melanogaster				
(Oregon-R)	♂	25°/70%	LT50:47 (estimated)	Massie, *et al.*, 1976.
(Oregon-R)	♂	21°/not stated	$\bar{x}$:91.3; max.:128	Miquel, *et al.*, 1976.
	♂	27°/not stated	$\bar{x}$:42.1; max.:61(est.)	
(Oregon-R)	♂	27°/not stated	LT50:31; LT100:38 (estimated)	Robinson, *et al.*, 1976.
(iso ♀ lines nat. pops.)	♂ ♀	20°/not stated	$\bar{x}$ of all lines:76.7 $\bar{x}$ of all lines:83.6	Parsons, 1978a.
(Oregon-R)	♂	21 ± 0.5°/not stated	LT50:77; LT100:140 (estimated)	Economos, *et al.*, 1979.
(Oregon-R)	not stated	25 ± 0.5°/65 ± 10%	$\bar{x}$ life span: 30	Lints, *et al.*, 1979.
		Longevity studies using the above strains after long-term brother-sister matings have been excluded from this table.		
Other Species				
D. persimilis	not stated	15°/not stated	LT50:110; LT100:180 (estimated)	Spiess, *et al.*, 1952.
D. subobscura	♀	20°/not stated	LT50:72; LT100:96 (estimated)	Maynard Smith, 1962.
D. simulans	sexes comb.	25 ± 1°/not stated	$\bar{x}$ life span: 34.83	Felix and Ramirez, 1969.
D. virilis			$\bar{x}$ life span: 54.03	
D. melanogaster			$\bar{x}$ life span: 36.47	
D. pseudoobscura			$\bar{x}$ life span: 32.31	
D. grimshawi	♀ (1)	65–68°F/not stated	life span: > 9 mos.	Carson, *et al.*, 1970.
D. repleta	not stated	25°/not stated	$\bar{x}$ life span: 2 mos.	Sohal, 1970.
D. willistoni	not stated	22 ± 2°/30–50%	LT50:28; LT100:52	Armstrong, 1976.
D. simulans (iso ♀ lines nat. pops.)	♂ ♀	20°/not stated	$\bar{x}$ of all lines:58.1 $\bar{x}$ of all lines:59.8	Parsons, 1977.

*(comments): Estimated numbers are interpolated from life tables or survival curves in cited references.
LT50: Number of days when 50% of flies are no longer alive.
LT100: Number of days when 100% of flies are no longer alive.

in various laboratories, using different environmental regimes. These environmental factors, themselves, however, can influence longevity and preclude accurate comparisons (Lamb, 1979). Until life span data are collected under the same culture conditions for a variety of *Drosophila* species, a proper choice of species for comparative analyses of the aging process cannot be undertaken.

The objective of the present study is to compare life span characteristics in *Drosophila*

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using species respresenting both different degrees of phylogenetic relatedness and varying ecological backgrounds.

METHODS AND PROCEDURE

Animals

Drosophila species groups were chosen as representatives of each of the following ecological backgrounds:

1. Temperate-montane Forest: *virilis* group.
2. Desert: *repleta* group.
3. Cosmopolitan: *melanogaster* group.
4. Tropical Forest: *willistoni* group.

The *virilis*, *repleta* and *melanogaster* species groups are each represented by triads containing two sibling species and a third more distantly related species, while the *willistoni* species group is represented by a triad containing three semispecies (Table 2).

Housing

All species were housed in a chamber kept at 19–20°C, 67–70% RH, and constant light (88–92 microwatts/cm²). Flies were raised in one-half pint bottles with a raisin based culture medium (Rockwell and Seiger, 1973).

Experimental procedures

For each species, ten virgins of both sexes were collected on the day of eclosion, kept separately by sex in standard shell vials containing identical amounts of culture medium, and housed in the rearing chamber. Flies were

TABLE 2. DROSOPHILA SPECIES: PHYLOGENETIC AND ECOLOGICAL BACKGROUNDS (THROCKMORTON, 1975).

Subgenus	*Species Group*	*Species*	*Population Location*	*Phylogenetic Relationship*	*Ecological Background*
Drosophila	Virilis	virilis	Texmeluca, Mexico	Sibling Sp.	Temperate-Montane Forest
		americana	Poplar, Montana	Sibling Sp.	
		montana	Anchorage, Alaska	Non-sibling Sp.	
Drosophila	Repleta	arizonensis	Sonora, Mexico	Sibling Sp.	Desert
		mojavensis	Sonora, Mexico	Sibling Sp.	
		mulleri	Monterrey, Mexico	Non-sibling Sp.	
Sophophora	Melanogaster	melanogaster	Florida	Sibling Sp.	Cosmopolitan
		simulans	Georgetown, Guyana	Sibling Sp.	
		ananassae	Trinidad	Non-sibling Sp.	
Sophophora	Willistoni	paulistorum-Amazonian	Belem, Brazil	Semispecies	Tropical Forest
		paulistorum-Interior	Colombia	Semispecies	
		paulistorum-Transitional	Guatapo, Venezuela	Semispecies	

transferred to fresh vials at three to four-day intervals and the number of survivors in each vial was recorded. Five replicate vials were used for each sex, totalling 50 males and 50 females per species.

Data comparisons

Survival curves for both sexes of each species were plotted based on the mean values of five replicates. Other data include:

1. LT50: mean number of days (mean of five replicates) when 50% were no longer alive.
2. LT100: mean number of days when 100% were no longer alive.
3. Range: mean number of days between the deaths of the first and last flies.
4. Mortality Rate (regression coefficient): linear regression equations were determined using the angular transformation of percent survival as the dependent variable (Zar, 1974), and age in days as the independent variable. Data from five replicates were grouped, since this procedure has no effect on the regression coefficient (Freund, 1971).

Intraspecific comparisons

Within species, male–female comparisons of LT50, LT100, range and mortality rate were made using Student's t-test (see Zar, 1974, for use of t-tests comparing regression coefficients).

Interspecific comparisons

Among species, comparisons of males were made separately from comparisons of females. In addition, individual species were only compared to species of their own group triad. LT50, LT100, range and mortality rate were compared using the Student-Newman-Keuls test (SNK) after performing an analysis of variance for all 12 species (see Zar, 1974, for use of SNK comparing regression coefficients).

Intergroup comparisons

Among species group triads, comparisons of males and females were again made separately. LT50, LT100, range and mortality rate were compared using the above mentioned analyses of variance and SNK tests. Two species groups were considered statistically different only if there was no overlap in the SNK test among any species in either triad.

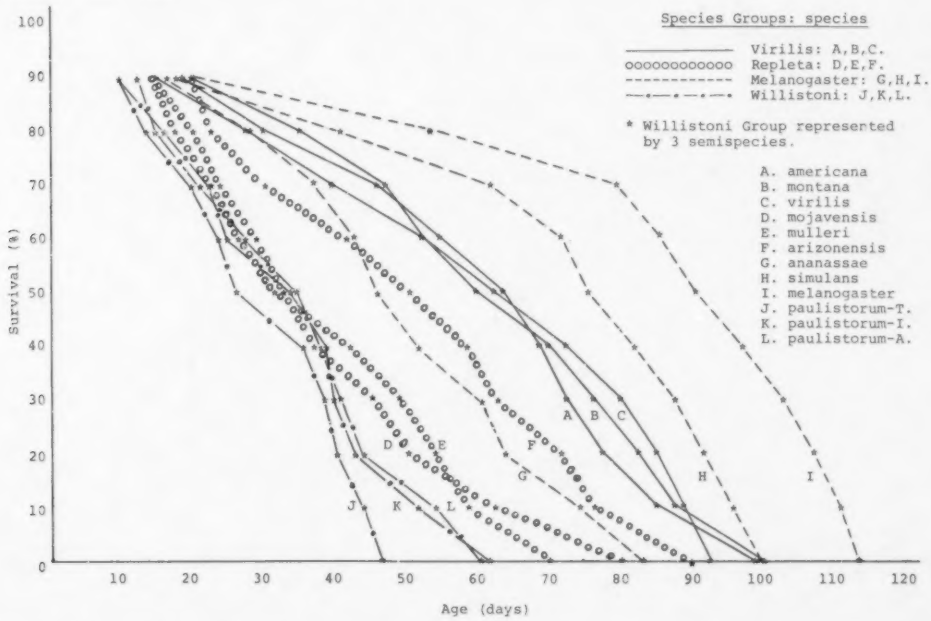

FIG. 1. Male survival curves of four Drosophila species groups: 12 species*.

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RESULTS

Survival curves

As seen in Figures 1 (males) and 2 (females), the survival curves of the 12 *Drosophila* species, raised under identical conditions, demonstrate the diversity in lifespan found in the genus. Within this diversity, there is clustering of species group triads in both sexes. The curves of the cosmopolitan melanogaster group triad seem to be exceptions, in that they do not cluster. Additionally, the shapes of survival curves vary. Most of the *melanogaster* and *virilis* group species have the commonly observed rectangular curve, whereas the curves of the *repleta* and *willistoni* group species are more linear.

Lifespan characteristics

Data comparing male and female lifespan characteristics in 12 *Drosophila* species are presented in Table 3.

Statistical comparisons

Tables 4 and 5 present the results of all statistical comparisons.

Intraspecific: males vs. females (Table 4-A). Within species, both sexes show similar lifespan characteristics. In 48 comparisons (12 species, 4 characteristics), significant differences were found in only 15 cases, with males showing a shorter lifespan than females. When males have significantly lower values than females in LT50, LT100 or range, there is usually a correspondingly higher value in their mortality rate (regression coefficient).

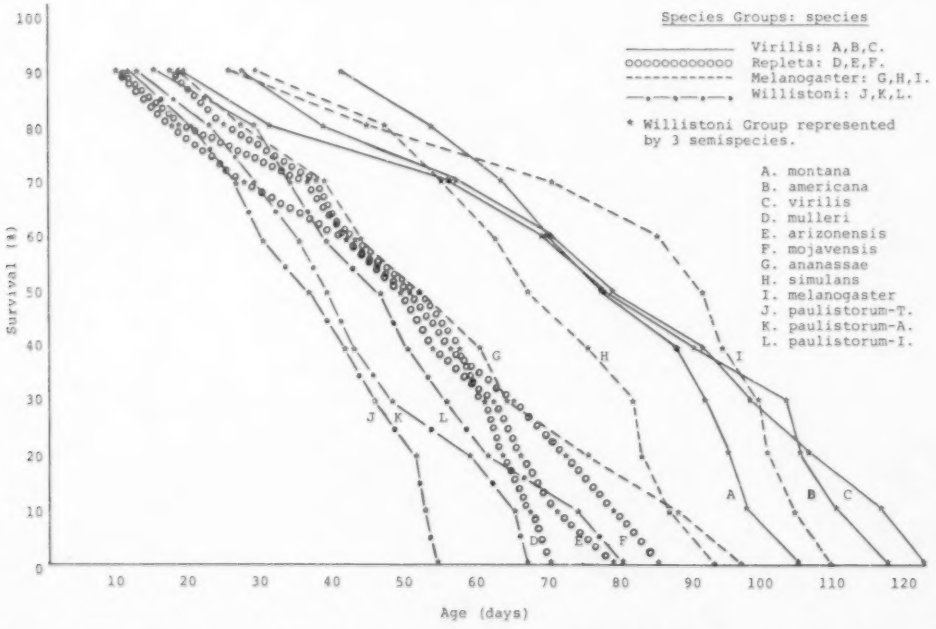

FIG. 2. Female survival curves of four Drosophila species groups: 12 species*.

TABLE 3. DROSOPHILA LIFESPAN CHARACTERISTICS.

Species	*Sex*	*LT50 ± S.D.*	*LT100 ± S.D.*	*Range*	*Mortality Rate: (Coefficient) of Linear Regression Equation.*
Virilis Group					
virilis	♂	62.2 ± 15.1	92.4 ± 10.8	77.6 ± 5.6	y = 1.517 − (.013x)
	♀	79.2 ± 15.4	122.6 ± 6.8	95.2 ± 30.3	y = 1.508 − (.009x)
americana	♂	63.8 ± 10.4	98.8 ± 8.3	80.0 ± 10.3	y = 1.600 − (.014x)
	♀	78.6 ± 21.6	117.6 ± 11.6	99.4 ± 20.9	y = 1.522 − (.010x)
montana	♂	60.4 ± 18.6	100.0 ± 14.8	80.0 ± 13.1	y = 1.573 − (.013x)
	♀	78.0 ± 11.1	105.0 ± 10.8	63.6 ± 17.1	y = 1.840 − (.014x)
Repleta Group					
arizonensis	♂	49.8 ± 7.7	89.8 ± 5.9	70.6 ± 6.4	y = 1.552 − (.015x)
	♀	49.0 ± 12.9	79.2 ± 14.3	69.4 ± 14.2	y = 1.450 − (.014x)
mojavensis	♂	32.8 ± 9.0	80.0 ± 16.9	66.0 ± 16.0	y = 1.385 − (.015x)
	♀	50.6 ± 9.4	85.4 ± 10.8	68.0 ± 14.4	y = 1.589 − (.015x)
mulleri	♂	31.2 ± 6.0	70.0 ± 3.0	56.0 ± 4.9	y = 1.496 − (.019x)
	♀	51.2 ± 7.2	70.8 ± 10.4	61.0 ± 7.5	y = 1.532 − (.017x)
Melanogaster Group					
melanogaster	♂	90.4 ± 10.3	113.4 ± 7.6	93.0 ± 7.9	y = 1.632 − (.010x)
	♀	91.6 ± 4.8	110.0 ± 11.8	84.2 ± 20.5	y = 1.669 − (.011x)
simulans	♂	75.6 ± 11.1	99.6 ± 7.1	81.4 ± 14.2	y = 1.600 − (.012x)
	♀	67.2 ± 18.0	93.2 ± 10.2	63.6 ± 24.6	y = 1.655 − (.014x)
ananassae	♂	45.6 ± 9.8	83.4 ± 19.7	66.6 ± 13.3	y = 1.488 − (.014x)
	♀	51.8 ± 16.7	96.8 ± 18.5	77.8 ± 15.0	y = 1.484 − (.012x)
Willistoni Group					
paulistorum-Amazonian	♂	34.0 ± 8.5	60.2 ± 12.2	48.2 ± 6.1	y = 1.454 − (.020x)
	♀	39.2 ± 18.4	67.2 ± 10.5	54.6 ± 6.8	y = 1.427 − (.016x)
paulistorum-Interior	♂	32.8 ± 5.3	61.6 ± 14.8	51.8 ± 13.2	y = 1.374 − (.017x)
	♀	47.0 ± 17.6	80.6 ± 13.9	65.4 ± 5.8	y = 1.479 − (.015x)
paulistorum-Transitional	♂	25.6 ± 5.1	46.8 ± 5.3	37.0 ± 10.4	y = 1.541 − (.027x)
	♀	36.2 ± 6.3	54.8 ± 9.1	43.6 ± 10.7	y = 1.557 − (.022x)

This is not unexpected if they are shorter-lived (Maynard Smith, 1962), however, these two tendencies are not always present concurrently. For example, in *D. mojavensis,* males have a shorter LT50 than females, yet their mortality rates are not significantly different. Similarly, males of *D. montana, D. simulans, D. ananassae, D. paulistorum*-Amazonian and *D. paulistorum*-Interior all have statistically different mortality rates from conspecific females, yet no differences in other male–female comparisons were observed. It is also of note that in *D. simulans,* the females have the higher rate of mortality.

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TABLE 4. STATISTICAL COMPARISONS: WITHIN SPECIES GROUPS.

Lifespan Characteristic	*A. Intraspecific (♂ vs. ♀)*	*B. Interspecific (♂)*	*C. Interspecific (♀)*
Virilis Group			
LT50	no significant differences	no significant differences	no significant differences
LT100	*vir.* ♂ < ♀: t = 5.28, p > .001 *amer.* ♂ < ♀: t = 2.95, p > .02	no significant differences	no significant differences
Range	no significant differences	no significant differences	*amer.* > *mont.*: p > .05 (SNK)
Mortality Rate	*vir.* ♂ > ♀: t = −7.43, p > .001 *amer.* ♂ > ♀: t = −13.86, p > .001 *mont.* ♂ < ♀: t = 3.02, p > .01	no significant differences	*vir.* < *mont.*: p > .01 (SNK) *amer.* < *mont.*: p > .01 (SNK)
Repleta Group			
LT50	*moj.* ♂ < ♀: t = 3.05, p > .02 *mull.* ♂ < ♀: t = 4.78, p > .001	no significant differences	no significant differences
LT100	no significant differences	no significant differences	no significant differences
Range	no significant differences	no significant differences	no significant differences
Mortality Rate	*mull.* ♂ > ♀: t = −3.28, p > .01 *ariz.* ♂ > ♀: t = 2.60, p > .02	*ariz.* < *mull.*: p > .01 (SNK) *moj.* < *mull.*: p > .01 (SNK)	*ariz.* < *mull.*: p > .05 (SNK)
Melanogaster Group			
LT50	no significant differences	*mel.* > *sim.*: p > .05 (SNK) *mel.* > *anan.*: p > .01 (SNK) *sim.* > *anan.*: p > .01 (SNK)	*mel.* > *anan.*: p > .01 (SNK)
LT100	no significant differences	*mel.* > *anan.*: p > .01 (SNK)	no significant differences
Range	no significant differences	*mel.* > *anan.*: p > .01 (SNK)	no significant differences
Mortality Rate	*anan.* ♂ > ♀: t = −3.62, p > .001 *sim.* ♂ < ♀: t = 2.79, p > .01	*mel.* < *anan.*: p > .05 (SNK)	*mel.* < *sim.*: p > .01 (SNK)*
Willistoni Group			
LT50	*paul.*-T ♂ < ♀: t = 2.93, p > .02	no significant differences	no significant differences
LT100	no significant differences	no significant differences	*paul.*-I > *paul.*-T: p > .05 (SNK)
Range	no significant differences	no significant differences	no significant differences
Mortality Rate	*paul.*-A ♂ > ♀: t = −7.28, p > .001 *paul.*-T ♂ > ♀: t = −6.08, p > .001 *paul.*-I ♂ > ♀: t = −3.66, p > .001	*paul.*-A > *paul.*-I: p > .01 *paul.*-A < *paul.*-T: p > .01 *paul.*-I < *paul.*-T: p > .01 (SNK)	*paul.*-A < *paul.*-T: p > .01 *paul.*-I < *paul.*-T: p > .01 (SNK)

*See text.

Interspecific: within species group triads (Table 4-B, C). Species within group triads also show similar life span characteristics. Within sex comparisons reveal only 20 statistically significant differences out of a possible 96. Here, genetic relatedness appears to play a

role, in that only one comparison shows a greater difference between two sibling species, than between one or both siblings and the third species of the triad (the mortality rates of *D. melanogaster* and *D. simulans* females are different from each other, yet neither species differs from the females of *D. ananassae*).

Within the *willistoni* triad of semispecies, we do not find any more similarity than within triads consisting of full species. The pattern of differences suggests that *D. paulistorum*-Amazonian and *D. paulistorum*-Interior are more similar to each other than to *D. paulistorum*-Transitional.

Table 4-B, C also indicates that differences among species may be found in only one or in both sexes. *D. americana* and *D. montana* females differ in their ranges and mortality rates, yet males do not. Similarly, *D. mojavensis* and *D. mulleri* males differ in their mortality rates, yet females do not. In contrast, *D. melanogaster* and *D. ananassae* show the same degree of difference in LT50 when comparing both males and females. This inconsistency in pattern underscores the danger in using one life span characteristic to predict results for others or in assuming that results found in one sex will apply to the other.

When the regression coefficient and any other life span characteristic are *both* significantly different among two species, the shorter-lived species always has the higher mortality rate (see *D. americana* vs. *D. montana* females; *D. paulistorum*-Interior *vs.* *D. paulistorum*-Transitional females). As in male-female comparisons (Table 4-A), a difference among two species in only one of these characteristics does not necessitate a corresponding difference in the other.

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Intergroup: among species group triads (Table 5). As was seen in male-female and within group comparisons, similarity in life span characteristics is predominant among species groups. Examination of males and females separately in each of the four life span categories permits 48 intergroup comparisons, of which only seven were found statistically significant. Five cases involve the *virilis* and *willistoni* groups, triads from different subgenera, which clearly have very different survival curves (Figures 1, 2). Yet, even these two species groups are not statistically different in all investigated life span characteristics. The other two cases involve triads from the same subgenus.

TABLE 5. STATISTICAL COMPARISONS: AMONG SPECIES GROUPS.

Lifespan Characteristic	*Males*	*Females*
LT50	Virilis Gp. > Willistoni Gp.: $p > .01$ (SNK)	Virilis Gp. > Willistoni Gp.: $p > .05$ (SNK) Virilis Gp. > Repleta Gp.: $p > .05$ (SNK)*
LT100	Virilis Gp. > Willistoni Gp.: $p > .01$ (SNK) Melano. Gp. > Willistoni Gp.: $p > .05$ (SNK)*	Virilis Gp. > Willistoni Gp.: $p > .05$ (SNK)
Range	Virilis Gp. > Willistoni Gp.: $p > .01$ (SNK)	no significant differences
Mortality Rate	no significant differences	no significant differences

*Species groups within the same subgenus (see text).

In summary, we find that when examining particular life span characteristics at three levels of comparison (intraspecific: male-female; interspecific: within species group triads; intergroup: among species group triads), similarities outnumber differences. When differences are found within triads, data support the idea of greater similarity between more closely related taxa. At all levels of comparison, when significant differences are observed in *both* the regression coefficient and any other life span characteristic, the shorter-lived subjects always have the higher mortality rate. However, differences cannot be used to predict from one characteristic to another or from one sex to the other.

DISCUSSION

In their description of Hawaiian species of *Drosophila,* Carson, *et al.* (1970) reported that *D. grimshawi* can live in excess of nine months. This finding suggests that a broad diversity in life span characteristics is present in *Drosophila* which, to date, has remained untapped in studies of the aging process.

The survival curves in Figures 1 and 2 and the data in Table 3 begin to reveal the range of lifespan characteristics in the genus. In addition, species groups appear to cluster within different intervals along the age continuum, indicating that representatives from different species groups can be useful for future comparisons. For example, the underlying basis of longevity differences can be examined using pairs of long-lived species such as *D. virilis* and *D. americana* or *D. melanogaster* and *D. simulans* in comparison with pairs of short-lived species from the *repleta* or *willistoni* groups. Similarly, whether mechanisms underlying longevity are the same in overlapping species from different species groups can be asked by comparing *D. mulleri* and *D. paulistorum*-Amazonian, or *D. ananassae* and *D. mojavensis.* Also of interest are the mechanistic bases of differences within a species group, such as those seen between *D. melanogaster* and *D. ananassae.*

There is an apparent conflict between the wide range of life spans and clustering of species groups depicted in Figures 1 and 2, and the overall similarities in life span characteristics revealed by subsequent statistical comparisons (Table 5). The paucity of statistical significance (7 of 48 comparisons) may be a function of our criterion for determining differences. By confining our definition of species group differences to cases where no overlap is permitted by any species of one group triad with any species in another group triad, we have set restrictions that mask the trends clearly shown in Figures 1 and 2. Although our criterion is theoretically sound, biologically it appears to be unrealistic.

Drosophila is a genus consisting of numerous subgenera, a minimum of 50 species groups, some of which contain up to 80 species (Throckmorton, 1975), and estimates of as many as 1500–2000 species (Stone, *et al.,* 1960). If there are biological limits on life span characteristics in *Drosophila,* it is not realistic to expect total non-overlap among all species, species groups and subgenera. Therefore, although the species group is useful for identifying species with strong similarities in lifespan characteristics (Table 4-B, C), it is too large a taxon to expect clear non-overlapping differences among such groups.

Phylogenetic versus ecological factors

The wide range of *Drosophila* life spans and the clustering of related species within that range raise the question, "Is the clustering of species groups (within group similarity) a result of the genetic relatedness of species within the group or a reflection of their

ecological backgrounds?" That is, since each species group also represents a different ecological background, it can be argued that the clustering of survival curves is not due to genetic similarity within the triad, but is simply a function of similar responses by the three species of each "ecological" group to the environmental conditions of the experiment.

The strongest evidence supporting the argument that genetic relatedness accounts for within group similarity is the fact that when differences are found between two species of a triad, only one case shows a difference between two sibling species (*: Table 4-C). Additionally, in the *willistoni* triad of semispecies, we observed that *D. paulistorum*-Amazonian and *D. paulistorum*-Interior are more similar to each other than either is to *D. paulistorum*-Transitional (Table 4-B, C), which is in agreement with the allozyme data reported by Richmond (1972,b).

Since semispecies are not fully reproductively isolated, one might expect them to be more closely related than full species, and therefore, more similar in lifespan characteristics. The number of differences found in the *willistoni* triad is not significantly less than the number found in triads containing full species. This need not be surprising, however, since Hubby and Throckmorton (1968) found that genetic identity between full species can be as much as 90%, which is not different from the average of 9.1% genetic difference attributed to semispecies (Richmond, 1972, b). We conclude that in terms of genetic relatedness, therefore, semispecies can be as similar or as different from each other as are full species.

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Our data also indicate that intraspecific male-female comparisons can be misleading when looking for phylogenetic similarities and differences in lifespan characteristics. A strict phylogenetic pattern should show fewer differences among sexes within a species than when comparing each sex separately among species. We found male-female differences within species to be more frequent (15/48 or 31.3%: Table 4-A) than in interspecific comparisons of the same sex (20/96 or 20.8%: Table 4-B, C). Numerous findings in the literature reveal male-female differences in life span characteristics (Table 1). When such differences were found here, males were shorter-lived. However, the use of additional populations may reveal the opposite to be true (Parsons, 1977, 1978, a). Furthermore, the higher than expected number of intraspecific male–female differences found in the present study confirm the statement by Samis, *et al.* (1971) that, ". . . differences between male and female *Drosophila* of different ages (cellular and subcellular) . . . show that the study of aging should be carried out separately for the two sexes."

An ecological interpretation of within group similarity and among group diversity in lifespan characteristics would focus on the optimality of environmental conditions in the present study (19–20°C) for each species. Parsons (1978, b) stated that differences between genotypes for longevity are magnified under stress compared with optimal environments. Similarly, a rectangular survival curve has been attributed to senescent deaths for populations living in an optimal environment (Miquel, 1971; Sacher, 1977; Lamb, 1979), which suggests that any shape other than rectangular, such as observed in the more "linear" *repleta* and *willistoni* groups, is a result of non-senescent deaths in a non-optimal environment. Based on these arguments, our data could be interpreted as reflecting differential degrees of stress/optimality imposed on each species by our experimental conditions.

We have additional data (manuscripts in preparation) concerning the ability to mate, oviposit and pupate at a range of temperatures (3–38°C), indicating that a "non-optimal environment" in the present aging study does not account for shorter longevity and linearly

shaped survival curves in all species reflecting these characteristics. Furthermore, Carson's *D. grimshawi* lived more than nine months at temperatures equivalent to ours (Table 1). The fact that *D. melanogaster* has never been reported to live as long, despite these temperatures being within its optimal range (Cohet, *et al.*, 1980), confirms that the ecological interpretation above does not adequately explain our findings. Thus, this raises the question as to whether there are different mechanisms of aging in *Drosophila*.

It is important to point out that previous assumptions regarding lifespan characteristics in *Drosophila*, such as rectangular survival curves, are based on experimental evidence derived from only one or two commonly used species. It is quite possible that a linearly shaped survival curve does not represent non-optimal experimental conditions, but instead, reflects different underlying aging mechanisms. An approach that incorporates the phylogenetic and ecological diversity in lifespan characteristics present in *Drosophila* is useful for addressing this and other aging questions. As our data indicate, however, species, sex and characteristic must be considered separately before firm conclusions can be drawn.

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SHORTENING OF THE *IN VITRO* LIFESPAN OF HUMAN DIPLOID FIBROBLASTS EXPOSED TO HYPERBARIC OXYGEN*

SHUJI HONDA and MITSUYOSHI MATSUO

Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho, Itabashiku, Tokyo 173, Japan

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Abstract—Serially cultivated WI-38 cells were exposed to 50 atm of oxygen for one hour at each passage after they reached 31 population doublings (PDs). They phased out at 51 PDs while the untreated and nitrogen-exposed cells at 56 PDs. The early-passage cells were found to be resistant to 50 atm of oxygen. The *in vitro* lifespan of WI-38 cells remained unchanged on less than eight exposures given before 46 PDs, and shortened in proportion to the number of extra exposures given after 46 PDs in addition to the seven exposures. For TIG-1 cells treated doubly with oxygen at late passages, modal cell volume began to increase at 54 PDs, and for the nitrogen-treated cells at 59 PDs. The *in vitro* aging of late-passage human cells exposed to hyperbaric oxygen seems to be accelerated.

INTRODUCTION

IT IS well known that human diploid cells have a limited proliferative capacity (Hayflick and Moorhead, 1961; Hayflick, 1965). This phenomenon is considered as a manifestation of cellular aging *in vitro* though its mechanism is still unclear. In the light of the present knowledge concerning cytogenetics, the *in vitro* aging of cells is likely to be under genetic control. However, it also can hardly be doubted that the *in vitro* aging advances under the influence of environmental factors; in fact, inclusion of hydrocortisone (Macieira-Coelho, 1966; Cristofalo, 1970) or epidermal growth factor (Rheinwald and Green, 1977) into culture medium and exposure to fluorescent light (Parshad and Sanford, 1977) extend the *in vitro* lifespan of human diploid cells and, on the other hand, exposure to ionizing radiation shortens their *in vitro* lifespan (Macieira-Coelho *et al.*, 1977; Ban *et al.*, 1980).

The high concentration of oxygen is thought to be a possible aging-accelerating agent on the basis of the observations that exposure to hyperbaric oxygen (Honda and Matsuo, 1980) or elevated partial pressures of oxygen (Rueckert and Mueller, 1960; Balin *et al.*, 1976) inhibited cell growth, and that the production of oxygen radicals, which are suggested to be the causative agents of aging (Harman, 1956), was enhanced in cells under hyperbaric oxygen (Fridovich, 1974). Previously, we found that the growth of late-passage human diploid cells was inhibited by exposure to hyperbaric oxygen more easily than that of early-passage cells (Honda and Matsuo, 1980). This implies that oxygen radicals possibly play a certain role in the aging process.

*Contribution TMIG-I No. 52.

In order to know whether or not hyperbaric oxygen can accelerate the *in vitro* aging of human diploid cells, we attempted to study the effect of exposure to hyperbaric oxygen on the lifespan of the cells. In the present paper, we describe that the *in vitro* lifespan of the late-passage cells exposed to 50 atm of oxygen shortened but that of the early-passage cells did not.

MATERIALS AND METHODS

Cell culture

Human diploid fibroblasts, WI-38 cells, were obtained from the Institute for Medical Research (Camden, NJ, U.S.A.) and another ones, TIG-1 cells, were established in our Institute (Ohashi *et al.*, 1980). Cells were grown in 3.5-cm plastic dishes (Lux Scientific Corporation, Newbury Park, CA, U.S.A.), each of which contained 2 ml of Eagle's basal medium (Grand Island Biological Company, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (GIBCO). Cultures were maintained at 37°C in a 5% CO_2-95% air atmosphere except during exposure to hyperbaric oxygen or nitrogen. Subcultivation was carried out weekly at either a 1:4 split ratio (WI-38 cells) or a density of 4×10^4 cells/dish (TIG-1 cells) by treatment with 0.25% trypsin (Difco Laboratories, Detroit, MI, U.S.A.) at 37°C for ten minutes. Medium was changed three days after inoculation. The number of cells was counted with a Coulter Counter Model ZBI (Coulter Electronics, Hialeah, FL, U.S.A.). Population doublings were estimated by the method of Good (1972). The ending of the lifespan of cells was defined as when cells did not grow over a one-week period. All cultures were free from mycoplasma (Kihara *et al.*, 1981).

Exposure to hyperbaric oxygen

Three days after inoculation, dishes containing cells were placed in a Parr's vessel (Parr Instrument Co., Moline, IL, U.S.A.). They were exposed for one hour to 50 atm of either oxygen or nitrogen which was compressed at a rate of 1 atm/5–6 seconds. Then, it was decompressed at a rate of 1 atm/9–10 seconds. Medium was renewed immediately after the exposure had finished.

Cell volume measurement

One week after inoculation, cells in each dish were detached with 0.25% trypsin and then suspended in 1 ml of medium. The cell suspension was diluted ten-fold with Isoton II (Coulter Electronics) at 0°C. Cell size was taken with a Coulter Counter Model ZBI (orifice: diameter 100 μm) equipped with a Channelizer Model C-1000 (Coulter Electronics) and calibrated using the standards of Microspheres (Coulter Electronics; diameters 9.58 and 20.43 μm).

RESULTS AND DISCUSSION

Serially cultivated WI-38 cells were exposed to 50 atm of oxygen for one hour at each passage after they reached 31 population doublings (PDs). As shown in Figure 1, they phased out at 51 PDs while the untreated and nitrogen-exposed cells at 56 PDs. A dye exclusion test with trypan blue revealed that very few of the oxygen-treated cells died out one day after exposure (data not shown). These findings suggest that the oxygen-induced lifespan shortening of human diploid cells results not from cell death, but from decrease in doubling potential. This presumption is supported by the fact that although the growth of WI-38 cells is inhibited by chronic exposure to elevated partial pressures of oxygen, most of them are viable (Balin *et al.*, 1976). In addition, we have observed that exposure to 50 atm of oxygen inhibited their growth (Honda and Matsuo, 1980).

Interestingly, on intermittent exposures, the early-passage cells were found to be resistant to 50 atm of oxygen. Figure 2 shows that the *in vitro* lifespan of WI-38 cells remained unchanged on less than eight exposures given before 46 PDs and shortened in proportion to the number of extra exposures given after 46 PDs in addition to the seven exposures. This seems to be related to the fact that with respect to colony-forming ability the late-

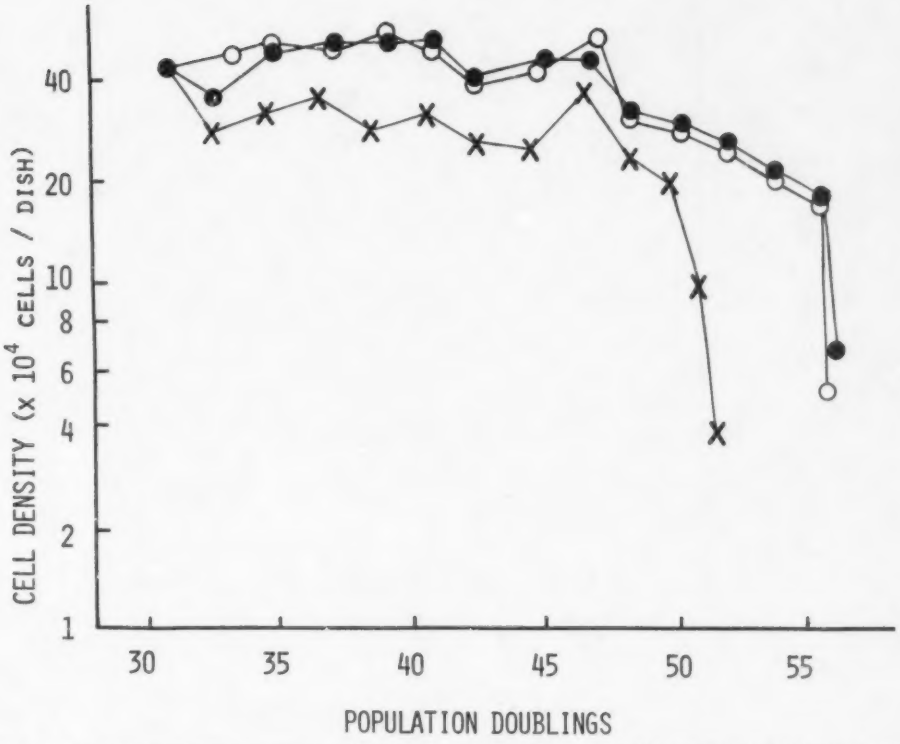

FIG. 1. Effect of intermittent exposures to 50 atm of oxygen on the cell densities and *in vitro* lifespan of WI-38 cells. The densities of oxygen-treated (×), nitrogen-treated (○) and untreated (●) cells were measured one week after inoculation. Oxygen exposures were carried out at each passage after 31 PDS.

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passage cells are more sensitive to oxygen than the early-passage cells (Honda and Matsuo, 1980).

In order to examine the lifespan shortening effect of oxygen on late-passage TIG-1 cells in detail, we compared the lifespan, cell density and cell volume distribution of the oxygen-treated cells with those of the nitrogen-treated cells.

As shown in Table 1, the *in vitro* lifespan of the late-passage cells exposed doubly to 50 atm of oxygen shortened but that of the early-passage cells did not. Since the cell densities of the oxygen- and nitrogen-treated late-passage cells cultured for one week after each subcultivation kept constant through the period from the completion of oxygen exposures to the onset of phase III, the aspects of growth of these cells appear to be similar, except that the oxygen-treated cells entered phase III earlier relative to the nitrogen-treated cells (Figure 3).

It is known that after human diploid cells have reached phase III, their cell size goes large and its distribution rapidly becomes heterogeneous (Simons, 1967; Cristofalo and Kritchevsky, 1969). Figure 4 shows changes in the cell size distribution of the oxygen- and nitrogen-treated late-passage cells during phase III. For the oxygen-treated cells modal cell volume began to increase at 54 PDs (Figure 4a) and for the nitrogen-treated cells at 59 PDs (Figure 4b); in the cell size distribution curves of the former after 59 PDs (Figure 4a) and of the latter after 63 PDs (Figure 4b), no peak corresponding to the modal cell volume

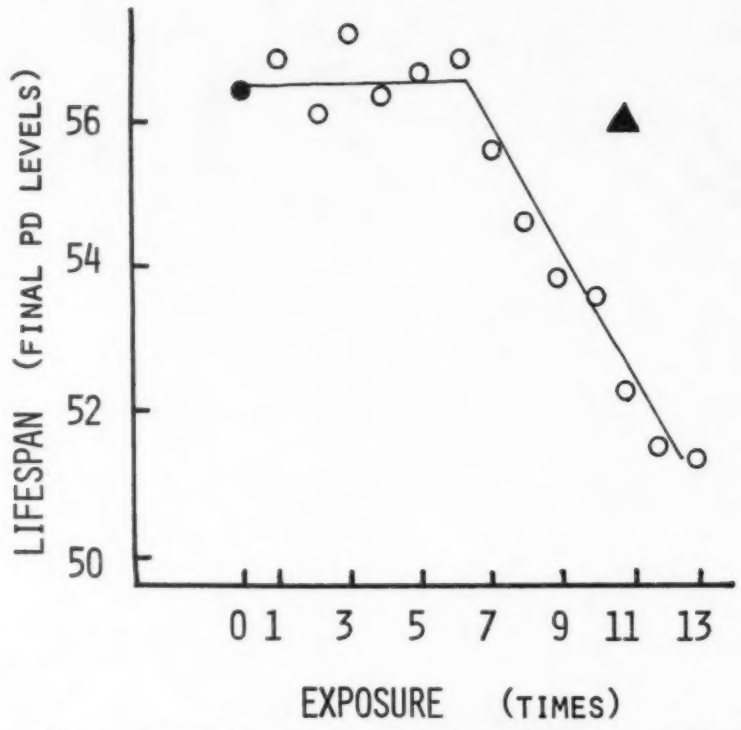

FIG. 2. Relationship between the number of exposures and the *in vitro* lifespan of oxygen-treated WI-38 cells. Exposures were conducted once a passage at successive passages after 31 PDs until desired exposures were achieved. The final PD levels of oxygen-treated (○), nitrogen-treated (▲) and untreated (●) cells are plotted as a function of the number of exposures. Each point represents the average value of 2 independent experiments.

TABLE 1. *IN VITRO* LIFESPAN OF TIG-1 CELLS EXPOSED DOUBLY TO 50 ATM OXYGEN.

Exposures[a]	*Final PD levels*[b]
N_2 (35 & 38)	66.0 ± 2.3[c] (6)
O_2 (35 & 37)	65.3 ± 1.9[c] (6)
N_2 (47 & 49)	64.1 ± 0.6[d] (5)
O_2 (47 & 48)	61.0 ± 1.1[d] (5)

[a]Cells were exposed to 50 atm of oxygen or nitrogen at the PDs in parentheses.

[b]Each value represents the mean ± the standard error followed by the number of determinations in parentheses.

[c]Insignificant differences between the final PD levels of the oxygen- and nitrogen-treated cells.

[d]Significant difference between the final PD levels of the oxygen- and nitrogen-treated cells ($p < 0.01$).

of a cell population became observable. Consequently, it is shown that the aging patterns of the oxygen-treated and control cells in phase III were quite similar and the *in vitro* aging of the late-passage cells exposed to hyperbaric oxygen was accelerated.

Harman (1956) put forward the free radical theory of aging that free radical reactions might contribute to aging deterioration in organisms. In regard to cellular aging *in vitro*, this theory seems to imply a mechanism underlying the lifespan shortening effect of ionizing radiation which enhanced free radical generation in cultured cells (Roots and Okada, 1972). Further, according to Fridovich (1974), oxygen radicals generated intracellularly due to hyperbaric oxygen are responsible for oxygen toxicity. It is, therefore, expected that the aging of cells exposed to hyperbaric oxygen is accelerated. The results reported here show that the *in vitro* lifespan of human diploid cells was shortened by oxygen exposures at late passages although it remained unchanged by oxygen exposures at early passages. These findings suggest that the *in vitro* aging of the late-passage cells whose defense function against oxygen radicals has deteriorated is accelerated by the radicals escaping from scavengers.

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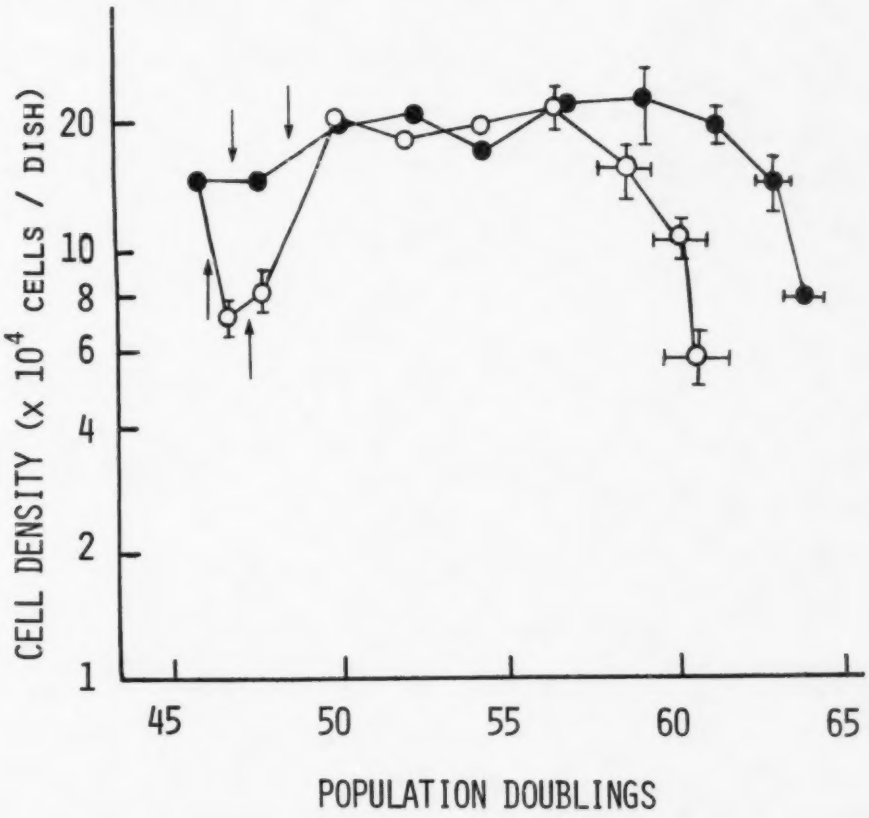

FIG. 3. Effect of oxygen exposures at late passages on the cell densities and *in vitro* lifespan of TIG-1 cells. Cell densities were measured one week after inoculation. Cells were exposed to 50 atm of oxygen at 47 and 48 PDs (○), or nitrogen at 47 and 49 PDs (●) as indicated by arrows. Each point represents the average value of 5 independent experiments. Each bar shows standard error.

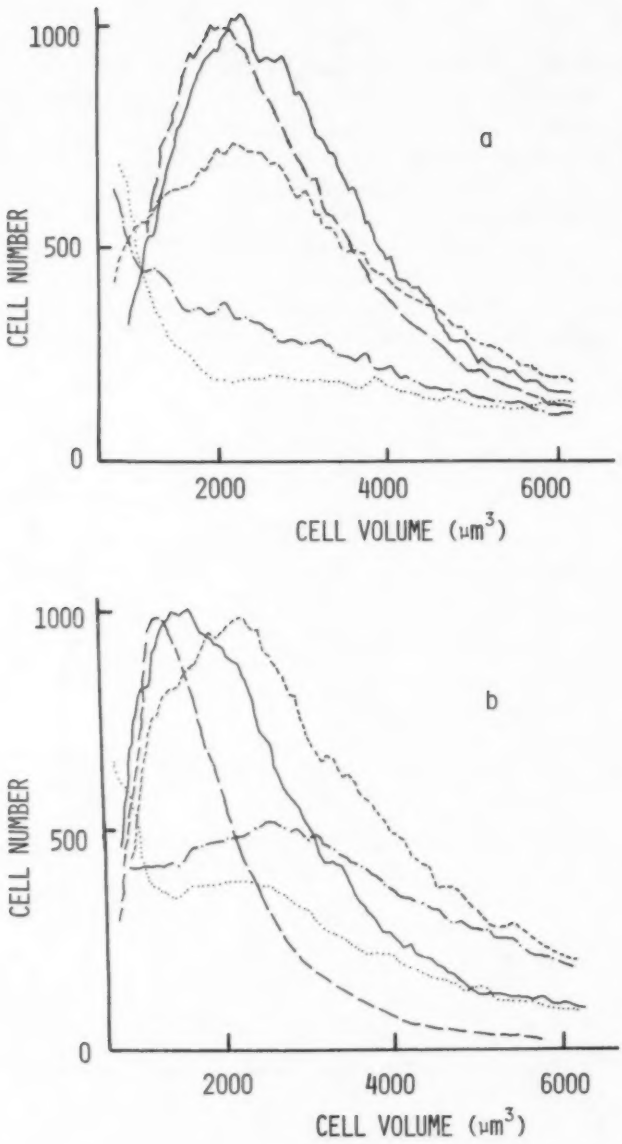

FIG. 4. Changes in the cell volume distribution of TIG-1 cells exposed to 50 atm of oxygen at late passages. Cells were exposed to 50 atm of oxygen (a) or nitrogen (b) as described in the legend to Fig. 3. Curves show the cell volume distributions of cell populations at the following PDs: (a) 54 (– –), 57 (——), 58 (----), 58.5 (– · –) and 59 PDs (·····); (b) 18 (– –), 59 (——), 61 (----), 62 (– · –) and 63 PDs (·····).

SUMMARY

The *in vitro* lifespan of serially cultivated human diploid fibroblasts exposed to 50 atm of oxygen at each passage shortened. Although the *in vitro* lifespan remained unchanged on less than eight exposures given at early passages, it shortened in proportion to the number of extra exposures given at late passages in addition to the seven exposures. From the experiments of oxygen exposure at early and late passages, it is evident that the *in vitro* aging of the late-passage cells exposed to hyperbaric oxygen was accelerated. To compare the aging patterns of the oxygen-treated and control cells, we examined changes in their cell densities and cell size distributions at each passage.

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DECREASING LEUKEMIA RISK IN OLD AKR MICE

PETER EBBESEN*, ÖRNULF BORGAN, and KNUT LIESTÖL

The Institute of Cancer Research (sponsored by the Danish Cancer Society), Radiumstationen, DK-8000 Aarhus C, Denmark
Institute of Mathematics, University of Oslo, Blindern, N-Oslo 3, Norway
Institute of Informatics, University of Oslo, Blindern, N-Oslo 3, Norway.

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Abstract—Leukemia incidence and survivorship were studied in 480 untreated adult female AKR mice using repeated blood testing on live animals and autopsy for diagnosis. The data were statistically analysed within the framework of a Markov illness-death model. The leukemia risk was close to zero below an age of four–five months, increased sharply to a maximum at eight–nine months, and then declined markedly. Old AKR mice therefore should be useful for studies of age dependent alterations that reduce the risk of leukemia development. The observed age-dependence of the leukemia risk is at variance with the simplest version of the multistep hypothesis. Allowing for individual tendencies for leukemia development, good correlation between expected and observed values can be obtained.

INTRODUCTION

MORE THAN 80% of AKR mice kept up to ten months of age develop leukemia (Staats, 1972). Therefore, these mice are frequently used for studying factors affecting the emergence of leukemia (Bortin and Truitt, 1977). The age-specific leukemia risk (= Leukemia intensity; see definition below) increases with age up to an age of eight–ten months. For AKR mice above this age, a previous study (Ebbesen, 1978) provided inconclusive evidence of a declining age-specific risk of leukemia. If confirmed, this decline in leukemia risk in old AKR mice would make them a suitable model for studies of age-dependent changes (or selection) which reduce the risk of leukemia development. The aim of the present study was to provide an accurate estimate of the age-specific risk of leukemia for untreated female AKR mice, and find a mathematical expression that fits the observations. Moreover, we wanted to evaluate a method to detect leukemia in live animals.

METHODS AND PROCEDURE

Inbred female SPF AKR mice originally obtained from Furth, and since 1958 maintained at the State Serum Institute, Copenhagen, were used. All mice initially aged 3 months were caged ten to a box and inspected six days

*To whom all correspondence should be addressed

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a week. Twenty mice were pre-selected to be killed at each of the ages 4, 5½, 7, and 9 months. Another 400 mice were observed to the age of 18 months, then the survivors were killed. All mice were palpated for enlarged lymph nodes and spleen and had eye blood drawn for leukocyte count and smear at the ages 4, 5½, 7, 9, and 13 months. Mice deemed dying or having reached the predetermined age for killing were studied in the same manner and then killed. At autopsy gross pathology and blood were examined, and lung, liver, spleen, thymus, and lymph nodes examined histologically. The diagnoses of leukemia based on palpation and blood examinations and those based on histology were carried out independently of each other.

The examinations based on palpation and leukocyte count and smear resulted in some obviously erroneous diagnoses of cancer. To minimize such errors, a "blind" re-examination of about 100 blood slides was carried out before the final analysis.

In ten randomly selected cases the leukemia diagnosis was confirmed by transplantation.

Statistical model and methods

The development of leukemia was analysed within the framework of the Markov illness-death model shown in figure 1. A mouse is born in the "box" labelled 0. If it gets leukemia, it moves to the "box" labelled 1. Upon death, a non-leukemic mouse moves to "box" 2, and a leukemic mouse to "box" 3. The intensity of contracting leukemia (= leukemia risk) is denoted $\sigma(x)$. That is, $\sigma(x) \cdot \Delta x$ is the probability that a mouse without leukemia of age x will develop leukemia in the small age span from x to $x + \Delta x$. Similar definitions apply to the forces of mortality [= death intensities $\mu(x)$ and $\nu(x)$]. In the analysis we assume that the intensities are constant over the age intervals 3–4 months, 4–5½ months, 5½–7 months, 7–9 months, 9–13 months and 13–18 months. Estimates of the age-specific intensities may then be found by standard maximum likelihood methods. When the number of animals is large, the estimates are approximately normally distributed (Borgan *et al.*, 1979).

RESULTS

The data are summarized in Table 1. The estimated intensities are given in Table 2, and the estimated leukemia risk for female AKR mice is illustrated in figure 2. The estimated leukemia intensity for female AKR mice shows a marked peak around seven–nine months. The fall in the leukemia intensity at higher ages is highly significant (Wald's test). For animals with leukemia, the death intensity is high and corresponds to a mean survival time of about two weeks after leukemia may be detected by examinations of blood. Death without leukemia occurred with a small intensity that increased with age. Mice dying without signs of leukemia had no evidence of other diseases.

TABLE 1. SURVEY OF DATA FROM EXPERIMENT WITH FEMALE AKR MICE

	Age in months					
Number of animals	*3–4*	*4–5½*	*5½–7*	*7–9*	*9–13*	*13–18*
Alive without leukemia[a]	477	425	287	118	31	6
Alive with leukemia[a]	1	10	15	13	1	1
Sacrificed without leukemia[b]	22	19	15	4	2	6
Sacrificed with leukemia[b]	0	0	3	6	0	1
Occurrence of leukemia, 0→1[c]	1	10	15	12	1	0
Death without leukemia, 0→2[c]	0	2	14	8	15	5
Occurrence of and death with leukemia, 0→3[c]	0	10	53	102	44	3
Death with leukemia, 1→3[c]	0	1	7	10	6	1
Disappeared or unknown disease status at death[d]	2	8	40	33	24	14

[a]Number of alive mice at the end of the age interval and before sacrifice.

[b]Number of preselected mice sacrificed at the end of the age interval.

[c]Number of occurrences during the age interval. The notation 0 → 1 etc. refers to figure 1.

[d]The number of animals that have disappeared or have unknown disease status at death. This is mostly due to cannibalism and the rapid decay of animals following death.

TABLE 2. ESTIMATED DEATH INTENSITIES AND LEUKEMIA RISK FOR FEMALE AKR MICE WITH ONE STANDARD DEVIATION

	Age in months					
Intensity	*3–4*	*4–5½*	*5½–7*	*7–9*	*9–13*	*13–18*
Death without leukemia	.000 –	.004 ±.003	.036 ±.009	.031 ±.010	.073 ±.018	.188 ±.053
Leukemia risk	.002 ±.002	.034 ±.007	.162 ±.018	.381 ±.033	.225 ±.032	.108 ±.053
Death with leukemia	–	1.050 ±.338	2.124 ±.309	2.354 ±.274	1.830 ±.295	.543 ±.322

As expected, histologic examinations detected leukemia at a somewhat earlier stage than examinations based on palpation and leukocyte counts plus leukocyte smears. This is illustrated in Table 3, which shows the comparability of these two methods of diagnosis when used at spontaneous death and at killing of pre-selected animals. To obtain a consistent definition of a "leukemic mouse" at the ages where diagnosis of live animals were carried out and at death, the above results have been based on diagnoses from leukocyte smear at the time of death.

We have also analysed the data by applying an extension of the model of Figure 1, allowing for separate states ("boxes") for "alive with leukemia detectable only by histological examinations" and "alive with leukemia detectable by examination of blood." The estimates of the transition intensity between these two states are unreliable. Nevertheless, the analyses showed that the results on the form of the leukemia intensity are only slightly affected by the uncertainty connected with the method of diagnosis. The estimated death intensity for leukemic mice depends on the method of diagnosis, but remains high irrespective of the method.

We have also re-analysed previously published histological material for about 600 untreated AKR controls (Ebbesen, 1978; Ebbesen and Doenhoff, 1971; Ebbensen, 1974a; Ebbensen, 1974b). Although the results from this re-analysis are quite uncertain, they confirm the results described above. The leukemia intensity for male mice is generally

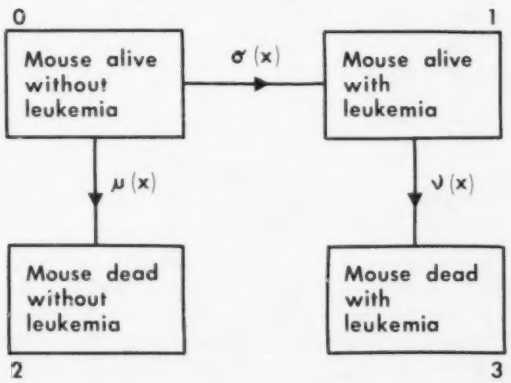

FIG. 1. A Markov illness-death model for the study of leukemia development for female AKR mice.

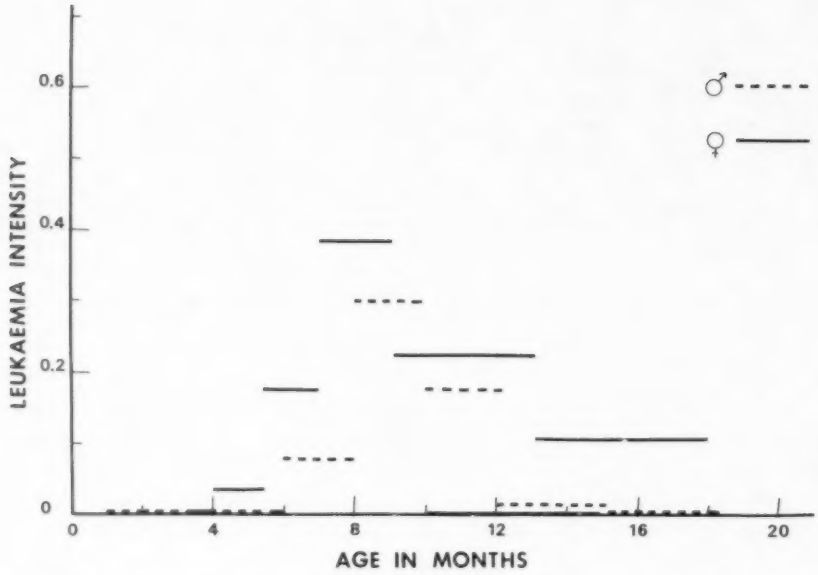

FIG. 2. Estimated leukemia intensity for female mice from the present experiment (——), and for male mice from previously published histological material (- - -). In the analysis we assume the intensities are constant over the age intervals indicated by the horizontal lines.

TABLE 3. COMPARISON OF PRE- AND POSTMORTEM DIAGNOSIS

A. MICE SACRIFICED AT PRESELECTED AGES		*Diagnosis based on histologic examination*	
		NON-LEUKEMIC	LEUKEMIC
Diagnosis based on palpation, blood counts and smears	NON-LEUKEMIC	52	5
	LEUKEMIC	1	8

B. SPONTANEOUSLY DEAD MICE AND MICE KILLED DUE TO IMMINENT SPONTANEOUS DEATH		*Diagnosis based on histologic examination*	
		NON-LEUKEMIC	LEUKEMIC
Diagnosis based on palpation, blood counts and smears	NON-LEUKEMIC	14	29
	LEUKEMIC	0	220

The number of mice given a certain diagnosis based on i) histology and ii) palpation plus leukocyte count and evaluation of the blood smear. The data are divided according to whether the mice were sacrificed (A) or not (B). Only mice diagnosed by both histology and evaluation of blood smear are included in the table.

Example: Upper horizontal row A) 57 (52 + 5) are judged non-leukemic based on palpation plus leukocyte count prior to sacrifice. Of these 57, 52 were found non-leukemic and 5 leukemic after histologic examination of the organs.

FIG. 3. Estimated leukemia risk for female AKR mice according to Table 2 (- - -) and fitted curve based on an inhomogeneity assumption (——). This last curve is based on a multi-step hypothesis where the intensity is assumed to be of the form $\sigma_a(x) = K \cdot (x - a)^n$. Here, n is an integer constant, x is age, a is the minimum age at which development of leukemia may start, and K is a random variable. K is assumed to be 0 with probability p, and to follow a gamma distribution $f(y) = (\alpha/k_o)^n \cdot y^{n-1} exp(-\alpha y/k_o)/\Gamma(\alpha)$ given that $K > 0$. Fitted parameter values: $n = 5$, $a = 1{,}59$, $p = 0.09$, $k_o = 1.1 \cdot 10^{-4}$, $\alpha = 0.71$.

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lower than for females, but expresses a similar peaked curve with a maximum at about eight–ten months (Figure 2). The estimated leukemia intensity for females in the reanalysis was even more peaked than the one shown in Figure 2 (Borgan *et al.*, 1979).

In female mice with abnormal lymphocytes at death, the average leukocyte count was about 60,000 (standard deviation 59,500), as compared to an (approximately age-independent) average of about 7,000 (standard deviation about 3,000) for live animals with no detectable leukemia. Mice examined about 14 days before they died with leukemia had leukocyte counts only slightly above the normal value, while the leukocyte counts on the average were doubled a week before death. During the last one–two weeks before death, there were also tendencies towards loss of weight (of the size order 3 g for a mouse of weight 25–30 g) and increase of palpable lymph nodes.

DISCUSSION

Present knowledge concerning the pathogenesis of murine leukemia indicates that there are a few probably randomly occurring steps that must take place. Endogenous C-type virus must be produced (Nowinski and Doyle, 1977; Hartley *et al.*, 1977; Chattopadhyay *et al.*, 1982), and infect somatic cells (Lilly *et al.*, 1975). Integration in cell DNA of the new virus-directed sequences seems to occur at random at many different sites on the hos-chromosome (Hughes *et al.*, 1978). According to one hypothesis, insertion adjacent to one of a few critical sites caused an enhanced expression of these genes (promotor effect), resulting in malignant transformation (Hayward *et al.*, 1981). Another prerequisite for AKR leukemia development is the normal differentation of bone-marrow stem cells into T lymphocytes (Mathieson *et al.*, 1978; Haran-Ghera, 1980; Wekerle *et al.*, 1980). Integration of virus-coded DNA in sites where transformation may be induced is probably more likely to occur at a certain stage of cellular differentiation (Boiver *et al.*, 1981).

Our estimated leukemia risk was similar when the diagnoses were based on blood slides and when the autopsy results were used. These results also agree with those obtained by the re-analysis of the previously published histological material. Thus, the estimated age-specific leukemia intensity for female AKR mice seems to be fairly precise. The estimate of the death risk for leukemic mice may be somewhat less precise, and depends on the applied method of diagnosis. The blood slide method detects the leukemia at a later stage than the autopsy method, and the estimated death rate will therefore be higher when the slide method is applied. Our data do not allow a reliable estimation of the delay in diagnosis caused by the blood slide method, but detectable infiltration in thymus, liver, and spleen clearly appear earlier than the rise in blood count and the presence of malignant cells in blood (Metcalf, 1962).

For simple versions of the multistep hypothesis, one may find mathematical expressions representing the age-dependence of the intensity. If it is assumed that the number of potentially malignant cells as well as the transition rates for each of the specific steps in each cell leading towards malignancy are the same for all mice and independent of age, then the leukemia intensity will (approximately) be of the form $\sigma_o(x) = k\,(x - a)^n$. Here, k and n are constants, x is age and a is the minimum age at which development of leukemia may start (Moolgavkar, 1978). Since $\sigma_o(x)$ is an increasing function of age, the declining leukemia intensity for older AKR mice is not in accordance with this form of the multi-step hypothesis. The leukemia intensity could be affected by age-related changes such as loss of cells capable of undergoing malignant changes (for example loss of thymocytes [Dunn, 1954], or age-dependent changes in immune response [Menuelo *et al.*, 1980]). Such age-related changes must be substantial in order to explain the difference between $\sigma_o(x)$ and the observed leukemia intensity. Another possibility is that some randomly occurring events, for example in connection with the integration processes of viral DNA mentioned above may take place in a limited age-span, thus creating a permanent difference between the animals (with respect to the number of potentially malignant cells or the transition rates in the multi-step process). Then the model for the multi-step hypothesis should be changed so that k in the expression for $\sigma_o(x)$ varies between the animals. To illustrate the possible effect of such a variation, we may use the following example: We assume that the value of k is, for each mouse, the outcome of a random variable, K. This random variable is assumed to be zero with probability p. Given that $K > 0$, we assume that the variable has a gamma distribution. The gamma-distribution may be written $f(y) = (\alpha/k_o)^{\alpha} y^{\alpha-1} exp(-\alpha y/k_o)/\Gamma(\alpha)$ where k_o *and* α are parameters, and $\Gamma(\alpha)$ denotes the gamma function. Now, the functional form of the leukemia intensity on the population level may be derived, and this function may be fitted to the estimates of Table 2 by the modified minimum chi-square method (Hoem, 1976). Figure 3 shows the result, and the model is seen to fit nicely. It should be noted that other distributions for the random K or other similar functional forms of $\sigma_o(x)$ (Moolgavkar and Knudson, 1981) also will give a reasonable fit. Thus, there are certain mathematically small and biologically plausible changes in the assumptions of the simple multi-stage model which imply a leukemia intensity similar to the one observed.

Each leukemic AKR mouse in the 6–12 month age group is known to harbor several antigenically distinct malignant T lympcocyte populations (Olsson and Ebbesen, 1979; Zielinski *et al.*, 1981). One explanation could be a sequential development of subpopulations of malignant cells due to chromosomal instability (Klein, 1980); alternatively, several normal lymphoid cell populations are susceptible targets for transformation. In

the latter case, the incidence at different ages of all leukemias lumped together can not be used for mathematical calculation of the number of steps necessary for leukemia development according to the original multistep hypothesis.

For the interpretation of the mathematical description of the observed data it is also important that there have been reports of old intact or thymectomized AKR mice which developed leukemia that in addition to T cell characteristics also had B cell markers (Mathieson *et al.*, 1982). We do not know the incidence of these leukemias with phenotype characteristics different from those of the leukemias developing at the "peak age." But clearly their presence means that the decline at high age in leukemia incidence among T cell leukemias must be even steeper than given by our curves.

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AGE-RELATED FUNCTIONAL ALTERATION OF MOUSE LIVER RIBOSOMES

J. VANDENHAUTE, N. CLAES-RECKINGER and J. DELCOUR

Laboratoire de Génétique Moléculaire et Physiologie Cellulaire Facultés Universitaires Notre-Dame de la Paix
B-5000 Namur, Belgium

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Abstract—The *in vitro* functional capacity of the mouse liver protein synthesis machinery was studied as a function of age. Polysomes from young (one–three months old) and old (18–24 months old) C57BL/6J mice were incubated under standard conditions in a ribosome-free reticulocyte lysate containing [^{3}H]-leucine. The incorporation of radioactivity into hot TCA-insoluble material was measured as a function of time and kinetic curves were compared. A drastic age-related decrease in the initial rate of leucine incorporation was observed when the total ribosomal fraction (containing the whole range of ribosomal aggregates including subunits and single ribosomes) was assayed. When "heavy polysomes" (fractions from which subunits and single ribosomes had been excluded) were compared, the same difference was observed. This latter result indicated that the observed alteration may be attributed to actively translating ribosomes. Results from experiments using inhibitors of initiation suggest that the observed age-related alteration can be attributed to a reduced capacity of ribosomes from older animals to sustain reinitiation.

LIST OF ABBREVIATIONS:
DOC = sodium deoxycholate
DTT = dithiothreitol
Hepes = N-2-hydroxyethyl piperazine N-2-ethane sulfonic acid
PMS = post-mitochondrial supernatant
TCA = trichloroacetic acid

INTRODUCTION

ON THE basis of numerous studies (for a recent review see Richardson, 1981) it is generally believed that the functional decline and the structural alteration observed in the senescent phenotype arise at the cellular level as a result of some impairment of genetic expression. Following Orgel's attractive hypothesis of aging, much work has been accomplished on the fidelity of this expression (Holliday and Tarrant, 1972; Popp *et al.*, 1976). Less work has been devoted to the quantitative aspect of gene expression although a reduction or

Correspondence and reprints requests should be addressed to: Prof. J. Delcour, Laboratoire de Génétique Moléculaire et Physiologie Cellulaire, Facultés Universitaires Notre-Dame de la Paix, Rue de Bruxelles 61, B-5000 Namur, Belgium.

even an unbalance in the output of protein synthesis could obviously have a disastrous outcome in terms of general homeostasis and survival capacity of the cell. To substantiate the hypothesis of an age-related change of the rate of protein synthesis, various attempts were made by means of measuring the rate of incorporation of labelled aminoacids *in vivo*. However, the data concerning protein synthesis in organs *in situ* or even in isolated cells remain conflicting (Du *et al.*, 1977; Ricca *et al.*, 1978; Beauchene *et al.*, 1970; Comolli *et al.*, 1972; Ove *et al.*, 1972). Much more coherent are the results from *in vivo* incubation experiments of postmitochondrial supernatants or microsomes in the presence of labelled aminoacids. In these conditions a drastic age-related reduction in proteosynthetic activity was observed (Layman *et al.*, 1976). As an attempt to define the functional defect causing this age-related reduction, polyribosomes from different classes of age were investigated. Results from this and other laboratories have previously shown that the average polysomal size is reduced as a function of age in rodent liver (Richardson *et al.*, 1981; Vandenhaute *et al.*, 1978; Layman *et al.*, 1976). However, a changed polysomal sedimentation profile alone cannot unequivocally be interpreted in terms of a changed output of proteins. The present investigation was undertaken in order to compare the *in vitro* translational performance of polysomes from young and old mice. We further investigated the effect of inhibitors of initiation on the leucine incorporation kinetics of these polysomes.

METHODS

Preparation of polysomes

Livers were obtained from young (1–3 months) and old (18–24 months) male C57BL/6J mice freely drinking and eating.

Polysomes were prepared according to the procedure described by Ramsey and Steele (1976) with minor modifications: Tris-HCl was used instead of Hepes; since only total polysomes were required, the PMS fraction obtained after low-speed centrifugation of homogenate (20,000 rpm, 4 minutes in a Sorvall SS34 rotor) and detergent treatment (DOC at 1.3% final concentration) was directly layered on top of a discontinuous sucrose gradient prepared as described in the original method (Ramsey and Steele, 1976); in addition, sucrose and sample volumes as well as run times were adapted for centrifugation in Beckman 65Ti rotor. The purified pellet of total polysomes was stored at −80°C for no more than one to two months until used.

For those experiments requiring only high-order polysomes, the pellets were resuspended and further fractionated by preparative sucrose gradient centrifugation (see below). The material from the disome-peak on to the bottom of the tube was collected and concentrated by precipitation in the presence of polyethylene glycol according to Expert-Bezançon *et al.* (1974).

Analysis of polysome size distribution

Samples of polysomes resuspended in buffer (50 mM Tris-HCl, pH 7.4, 75mM KCl, 5 mM Mg acetate and 3 mM DTT) were layered onto 15–50% (w/w) sucrose gradients prepared in the same buffer containing 0.5 mM EDTA and centrifuged for 110 min at 40,000 rpm (SW40, Beckman). After centrifugation the gradients were monitored continuously at 254 nm with an ISCO UA-5.

Cell-free protein synthesis

A commercially available reticulocyte lysate translation kit (NEN, n° NEK-002) was used to measure the translational activity of polysomes. All ingredients were used as recommended by the manufacturer except that prior to use the nuclease-treated lysate was centrifuged for 1 hour at 100,000 g (Beckman airfuge) in order to pellet out the reticulocyte ribosomes. A non-saturating amount of polysomes (15 μg) was incubated in a 25 μl volume at 25°C. At various time intervals 2 μl of incubation volume were withdrawn in order to measure the incorporation of [^{3}H]-leucine into hot TCA-insoluble material.

RESULTS

In vitro cell-free translation offers an accurate means to check the efficiency of the different components of the protein synthesis machinery under completely standardized conditions. In the present experiments a rabbit reticulocyte derived cell-free system freed from endogenous mRNA and ribosomes is provided with a non-saturating amount of mouse liver ribosomal fraction. Under these conditions, incorporation of aminoacids into newly synthesized proteins takes place on the natural mouse liver mRNA as a template. The time course of proteosynthesis is followed by measuring the incorporation of [^{3}H]-leucine into hot TCA insoluble material. In our conditions leucine incorporation is not far from steady state for about twenty minutes after which a plateau value is progressively reached, indicating that synthesis is arrested. Addition of fresh medium or pure mRNA at the plateau time has no effect, but addition of mRNA-free reticulocyte ribosomes induces proteosynthesis to resume (Figure 1). From this latter experiment it is concluded that in our conditions the ribosomal fraction is the limiting factor of the *in vitro* translational process. The kinetic curve is therefore an accurate tool for comparing the ribosomal efficiency in relation with aging.

Figure 2 which represents a typical leucine incorporation experiment shows that a severe reduction of the *in vitro* translational activity occurs as a function of age. Both the initial

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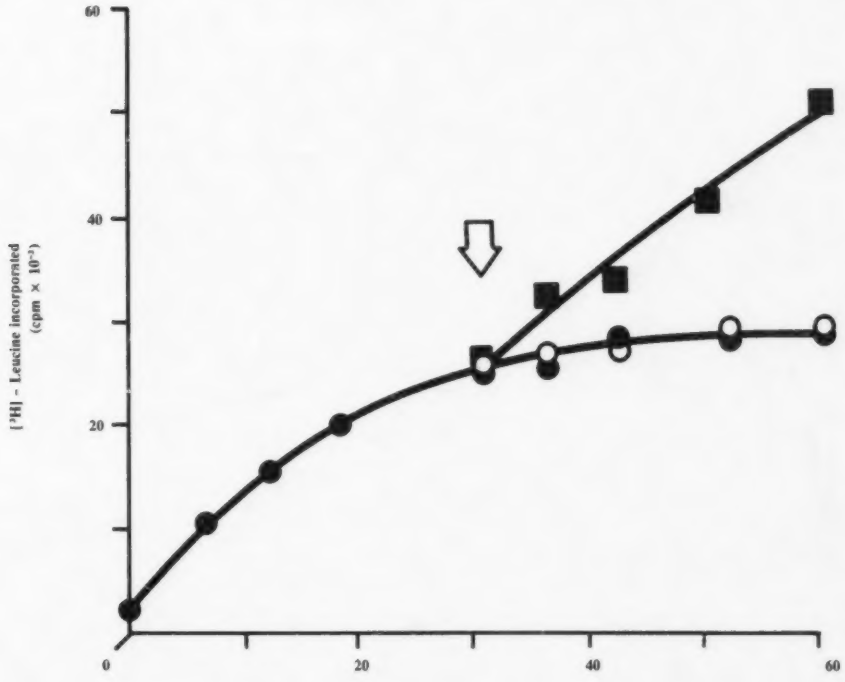

FIG. 1. [^{3}H]-leucine incorporation kinetics of hepatic polysomes. [^{3}H]-leucine incorporation was measured as described. After 30 min of incubation either 15 μg of reticulocyte ribosomes or 1 μg of globin mRNA were added. (● control; ○ addition of mRNA; ■ addition of ribosomes)

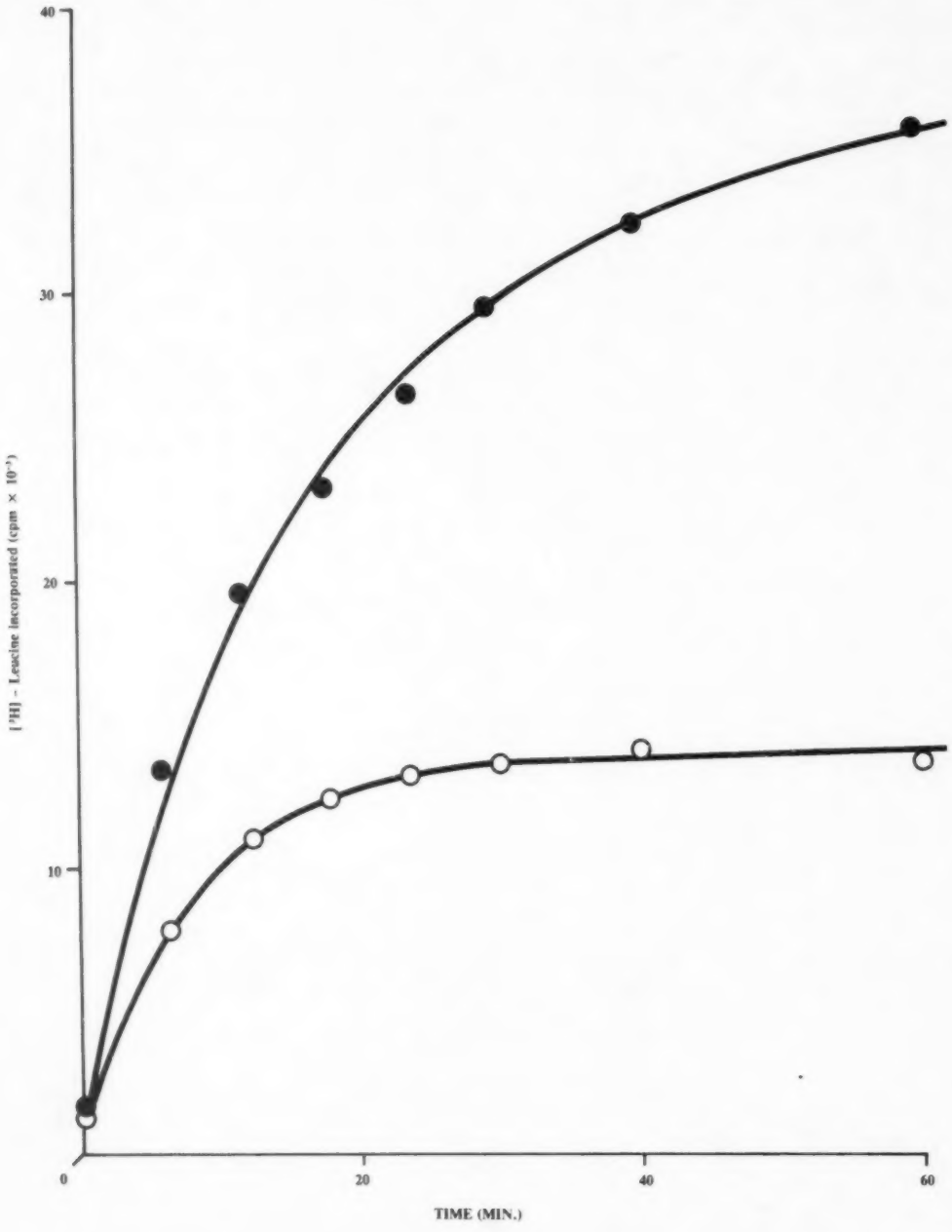

FIG. 2. Comparison of the *in vitro* protein synthesis capacity of hepatic polysomes from young (●) and old (○) mice. 15 μg of polysomes were incubated in a ribonuclease-treated reticulocyte lysate (freed from its endogenous ribosomes) in the presence of [³H]-leucine. At various time intervals 2 μl of the incubation volume (25 μl) were withdrawn and the incorporation of [³H]-leucine into hot TCA-insoluble material was measured.

TABLE 1. CHARACTERIZATION OF THE TIME-COURSE INCORPORATION OF [^{3}H]-LEUCINE INTO HOT TCA-INSOLUBLE MATERIAL USING LIVER POLYSOMES FROM YOUNG AND OLD MICE.

	young		*old*	
Experiment	α	*p*	α	*p*
1	855	36	561	14
2	820	25	640	16
3	669	32	436	16
4	768	34	216	10
5	560	25	261	10
6	649	24	408	16
Mean	720	29.3	420	13.7
Standard deviation	±113	± 5.3	±165	± 2.9

α: initial incorporation rate of [^{3}H]-leucine (cpm/min)
p: plateau value (cpm × 10^{-3})

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rate of synthesis and the plateau value are lower when ribosomal fractions from old mouse liver are used. Such a difference was reproducibly observed (Table 1).

The reduction of the overall activity exhibited by ribosomes from old animals could be due to the presence of inactive entities contaminating in a differential proportion the fractions from both age groups. In fact the analysis of the size distribution of polysomes by sucrose gradient sedimentation (as described above in the "Methods" section) provides a confirmation of our previous results (Vandenhaute *et al.*, 1978) and demonstrates a significantly higher proportion of single—presumably inactive—ribosomes in older cells as compared to young ones (Figure 3). These data account for the observed differences. In order to challenge this hypothesis we measured the *in vitro* translational activity of ribosomal fractions from which single ribosomes had been discarded; we call those fractions "heavy" polysomes. When comparing leucine incorporation kinetics of "heavy" polysomes from young and old animals we observe the same differences as in the experiments using total ribosomal fractions (data not shown). Thus the higher proportion of single ribosomes in old ribosomal fractions cannot be responsible for the observed age-related decrease of translational efficiency. As an alternative hypothesis we suggest that some impairment arises at the level of the functioning ribosome itself.

Initiation of translation is known to play a critical role in the control of the proteosynthetic activity in eukaryotic cells (Jagus *et al.*, 1981). In order to investigate whether an impairment of this process could explain the observed age-related reduction of the translation efficiency, we performed leucine incorporation experiments in the presence of inhibitors of initiation. Figure 4 shows the effect of heparin (1 mg/ml) on the incorporation kinetics of "heavy" polysomes from young and old mice. A drastic difference in sensitivity is observed. Whereas polysomes from old animals are almost unsensitive to the inhibitor of initiation, the incorporation kinetics of polysomes from young mice is greatly impaired: in the presence of heparin, it becomes similar to that of old animals, that is it exhibits a lower plateau value as well as a decreased initial rate of incorporation. We observe the same differential sensitivity when using two other inhibitors of initiation:

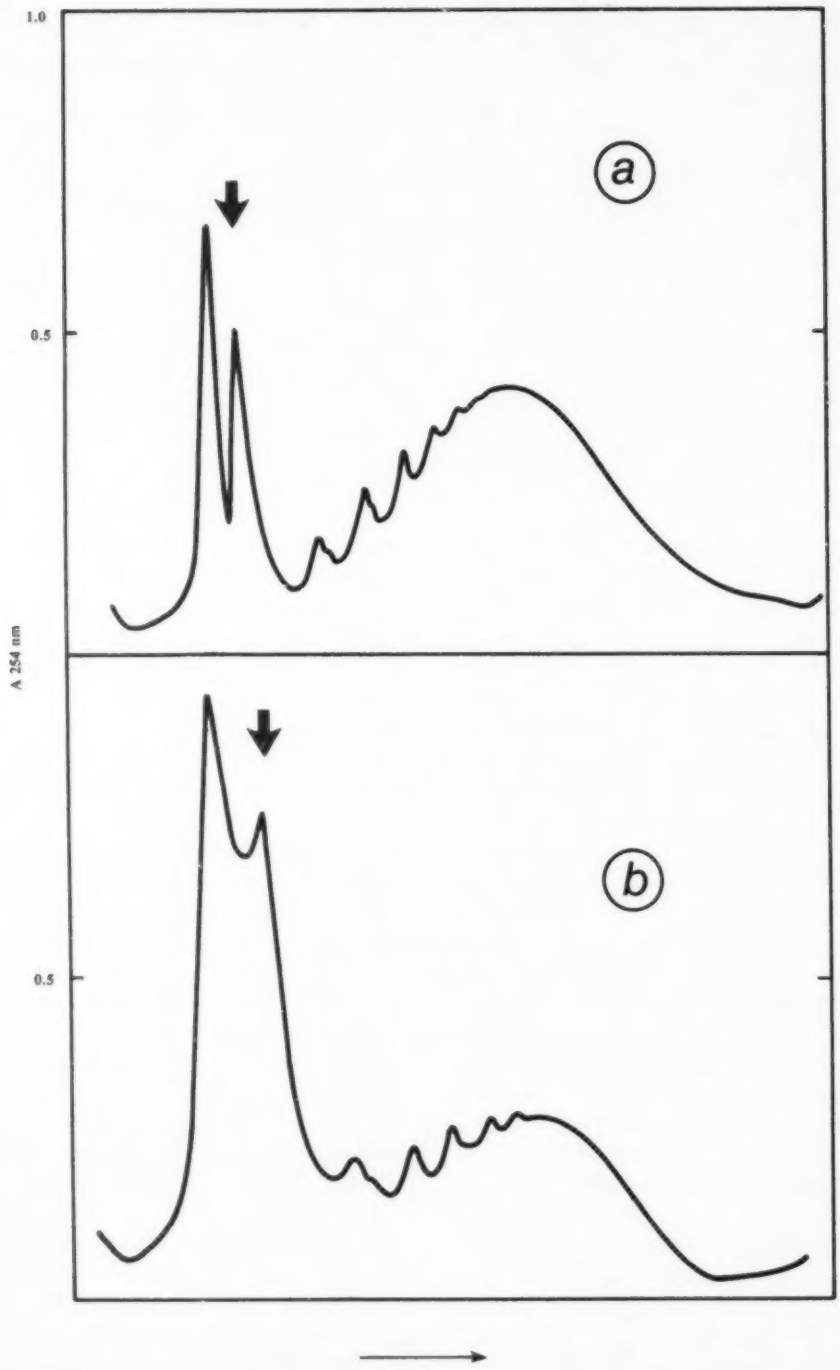

FIG. 3. Size distribution of purified liver polysomes from young (a) and old (b) mice. Polysomes were prepared and analysed as described in Methods. Direction of sedimentation is indicated by the horizontal arrow; the vertical arrow locates the 80S ribosomal peak.

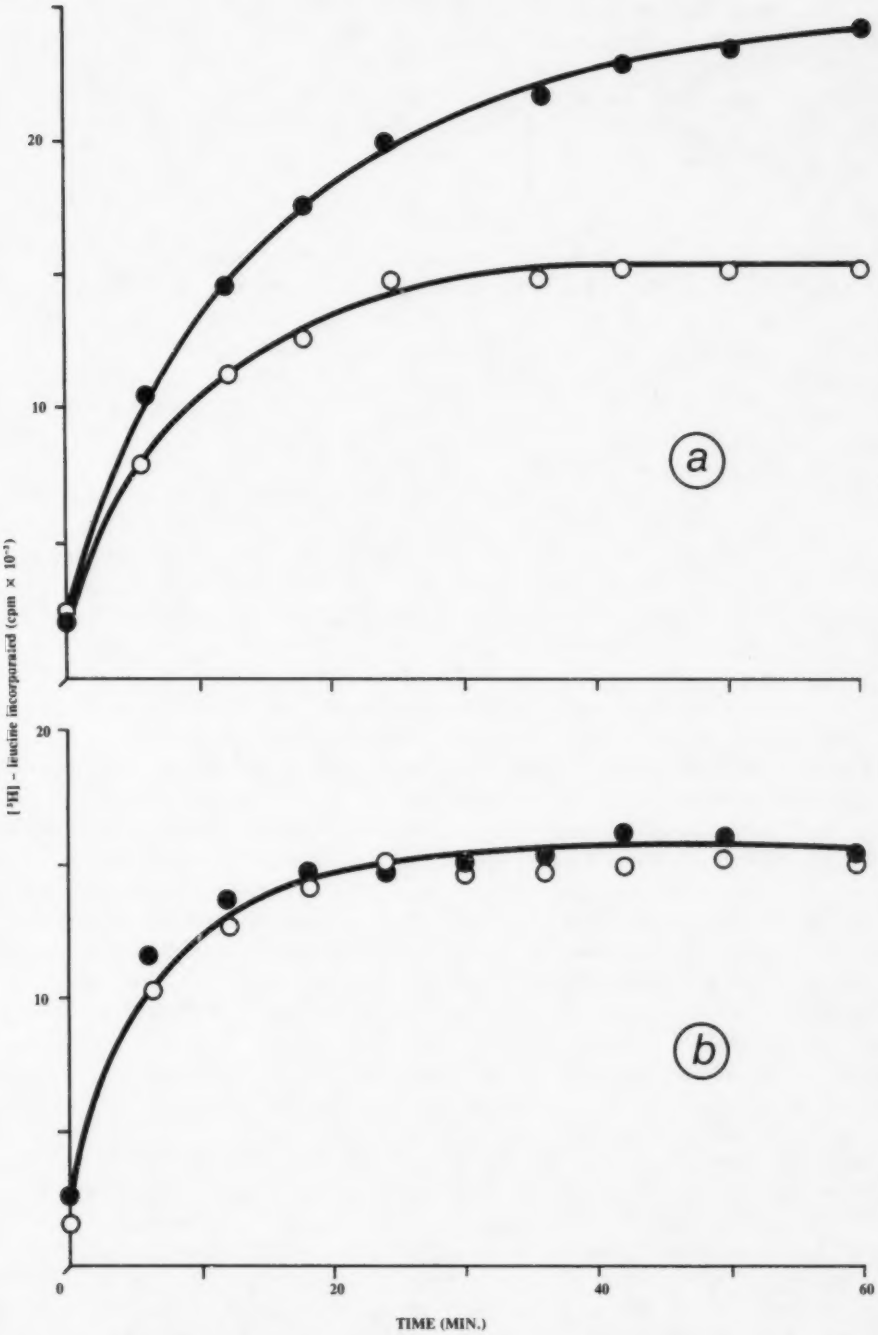

FIG. 4. Effect of heparin on leucine incorporation kinetics of polysomes from young (a) and old (b) mice. 15 μg of "heavy" (high-order) polysomes from mouse liver were incubated in a reticulocyte lysate. [³H]-leucine incorporated into hot TCA-insoluble materiai was measured as a function of time. (● control experiment; ○ in the presence of heparin)

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methylglyoxal and sodium fluoride (results not shown). These observations are consistent with the above-mentioned hypothesis of a reduced initiation capacity of ribosomes in older cells. Obviously, any impairment in the re-initiation process by run-off ribosomes will lead to a reduction in the initial rate of amino acid incorporation into proteins (both nascent and complete chains, as measured by hot TCA purification), as well as to a decrease in the plateau value.

DISCUSSION

The aim of this work was to investigate whether the functional capacity of the protein synthesis machinery in the mouse liver is modified as a function of age. We assayed the protein synthesis activity of purified polysomes in a nuclease-treated reticulocyte lysate. In these conditions the intrinsic ability of polysomes to synthesize proteins can be checked independently from other potentially important parameters mediating the regulation of proteosynthesis such as factors and cofactors, amino acid levels, tRNA and synthetases. When comparing leucine incorporation kinetics of polysomes from young and old animals, a significant age-related reduction of the *in vitro* performances is observed. Our results throw some light on previous findings from a number of *in vitro* studies suggesting an age-related impairment of proteosynthesis. Layman *et al.* (1976) have measured the protein synthesis capacity of rat liver post-mitochondrial supernatant. Their data suggest that the observed age-related decrease is associated with the ribosomal fraction. Buetow and Gandhi (1973) on the other hand conclude that in cell-free preparations from rat liver both cytosol and microsomes are involved in the observed differences. From our own data we conclude that polysomes are somehow impaired as a function of age.

Two possible causes of the age-reduced translational efficiency of polysomes have been considered: either the presence of a higher proportion of inactive ribosomes in ribosomal fractions from old animals or the existence of some general age-related functional defect which could reduce the overall translational ability of ribosomes in old animals. Analysis of the size distribution of polysomes from different age groups showed a shift towards lighter polysomes and a concomitant increase in the proportion of single monosomes occurring as a function of age. However, since we show that high-order polysomes (devoid of single ribosomes) exhibit the same age-related functional alteration as total polysomes we may exclude the first hypothesis. Moreover, we show that ribosomes, and not mRNA, are able to induce protein synthesis to resume after the plateau has been reached. Obviously, at least part of the age-related impairment of the protein synthesis machinery can be attributed to the functioning ribosome itself.

In order to assign this functional defect to a precise step of the translational cycle, we compared the effect of inhibitors of initiation on leucine incorporation kinetics of polysomes from young and old mice. The three inhibitors used—heparin, sodium fluoride and methylglyoxal—led to the same observations: polysomes from old mice gave identical kinetics irrespective of the presence of the drug, whereas in young animals inhibition of initiation brought down the kinetics to the level of old ones. From these experiments we conclude that the reduced *in vitro* efficiency of ribosomes from old animals is primarily due to an impairment of the initiation capacity. Our data do not provide any direct evidence as to the precise nature of the structural modifications involved in the observed functional alteration. However, it should be stressed that the preparative procedure of Ramsey and Steele (1976) for polysomes purification (centrifugation through a bilayer of dense sucrose containing 250 mM KCl) provides highly purified ribosomes. It seems

therefore unlikely, although not formally excluded, that the observed functional impairment could result from some differential contamination of the polysomes with soluble factors. Experiments are in progress to elucidate further the underlying molecular mechanisms.

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AGE-RELATED PROTEIN CHANGES IN THE BANANA FRUITFLY

I. JIT. and S.P. SHARMA*

Department of Biology, Guru Nanak Dev University, Amritsar-143005 India

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Abstract—Protein analyses from head and body revealed changes in content and electrophoretic banding pattern during aging of the banana fruitfly, *Zaprionus paravittiger*. Total protein content of head and body increased during the first 13 days of post-emergent life, followed by decline with advancing age. Electrophoretic studies indicate that certain proteins present in newly emerged flies disappeared with the advancing age while a few specific proteins appear and accumulate as the total protein content decreases during post-reproductive period.

INTRODUCTION

AGING, A POST-reproductive process, includes degradative changes in structure and function of an organism accompanied by many biochemical changes at cellular and subcellular levels (for example Rockstein and Miquel, 1973). Alterations in macromolecular metabolism are important manifestations of aging (Adelman, 1977). Decreased protein content and decreased capacity for protein synthesis during aging have been reported in some insects (Baumann, 1969); Baumann and Chen, 1968; Sharma and Sharma, 1979; Utsami and Natori, 1980). However, Clarke and Maynard Smith (1966) have observed an increase in the rate of protein synthesis with age in *Drosophila subobscura*. No change in protein content was reported by Lang *et al.* (1965) and Burcombe and Hollingsworth (1970). The present study is aimed to determine both quantitative and qualitative changes with age in banana fruitfly *Zaprionus paravittiger* to establish protein-aging relationships.

MATERIAL AND METHODS

The cultures of *Z. paravittiger* were raised and maintained on an artificial diet of 26 ± 2°C (Sharma and Jit, 1980). The mean life spans of female and male fruitflies are 38 and 34 days respectively (Sharma and Jit, 1982).

Age-related protein analysis was done using heads and bodies (= Thorax + abdomen) of insects, males and females, separately, of different age groups. The samples were homogenized in ground glass homogenizer at 0°C for five minutes in 0.1 M phosphate buffer (pH 7.2) containing 0.85% NaCl to obtain 5% homogenates for quantitative analysis and 10% homogenates for qualitative analysis. Each sample included 250 insects.

For estimation of protein content the homogenates were treated by the method of Burcombe and Hollingsworth (1970). The total protein content was measured by the method of Lowry *et al.* (1951). The standard curve was made using bovine serum albumin obtained from the Biochemical Unit, V.P. Chest Institute, Delhi-7.

The qualitative analysis of proteins was done by polyacrylamide gel electrophoresis at 4°C following the method of Ornstein (1964) and Davis (1964), but the concentration of gel used was ten percent. Homogenates

*To whom correspondence should be addressed

(10%, w/v) of tissues were centrifuged at 12,000 g for 20 minutes at −4°C to get soluble proteins. The extracts were not stored (−8°C) more than 24 hours. A constant volume of 0.1 ml of the extract was loaded to each gel tube. The experiments were carried out thrice in three generations of insects.

RESULTS

The protein contents of both heads and bodies increased initially during the first 13 days of post-emergent life, followed by gradual decline till the death of both sexes (Figures 1–4). There was not much significant difference in the total protein content of heads and bodies of the two sexes.

The electrophoretic analysis is represented in Tables 1–4 and in Figures 1–4. On the basis of increasing intensity, three types of bands, that is trace (+), minor (++), and major (+++), were characterized. In the zymogram of head proteins of freshly emerged flies, 23 and 22 bands were observed in females and males respectively. Body protein pattern of freshly emerged females and males showed 25 and 23 protein bands respectively. Protein pattern of flies of different age groups revealed that seven bands in female head protein pattern, 11 bands in male head protein pattern, eight bands in female body protein pattern and nine bands in male body protein pattern disappeared during adult aging. In the same period six new protein bands in female head and body protein patterns and five new protein transients in male head and body protein patterns appeared. Many of the protein bands were present throughout the life span, but some of them showed decrease in intensity.

DISCUSSION

Protein content of heads and bodies showed an initial rise and a subsequent decline with advancing age in both the sexes. Apparently, peaks of reproductive activity and protein content synchronize as the maximum egg laying rate is found between 8–14 days of adult life (unpublished data). These observations were comparable to those of most other insects (Samis *et al.*, 1971; Blevins, 1973). The protein contents of old flies (41 day-old) of both sexes is comparatively lower than that of the freshly emerged and reproductively matured flies, suggesting decreased protein synthesis or increased protein degradation in senescent flies. Decrease in protein contents with advancing age has earlier been substantiated by Baumann and Chen (1968), Baumann (1969), and Samis *et al.* (1971) in *D. melanogaster,* Blevins (1973) in *Phormia regina,* Rockstein and Baker (1974) in *Musca domestica,* Sharma and Sharma (1979) in bruchids and Utsami and Natori (1980) in *Sarcophaga peregrina.* Bender *et al.* (1970) and Kanungo *et al.* (1970) considered reduced protein synthesis and reduced RNA content to be a feature of aging process. Bergsma and Harrison (1978) ascribed the decreased levels of proteins with increasing age to decreasing number of genes coding for ribosomal RNA (rRNA).

Electrophoretic patterns of proteins from heads and bodies of flies of different age groups revealed that protein pattern of young flies included many distinct protein bands which were absent from protein pattern of old flies. Many new bands appeared during the age interval of one to nine days (might be significant for reproduction) and some new bands appeared during the period from 29 to 41 days, while some of the bands disappear during old age. The decrease in intensity of some of the bands cause increase in number of trace bands, which represents decreased protein content in old insects. Quantitatively the protein content (mg/50 mg fresh weight) decreased from 3.695 ± 0.055 and 3.689 ± 0.084 in 13 day-old insect bodies to 2.588 ± 0.088 and 2.233 ± 0.107 in 41 day-

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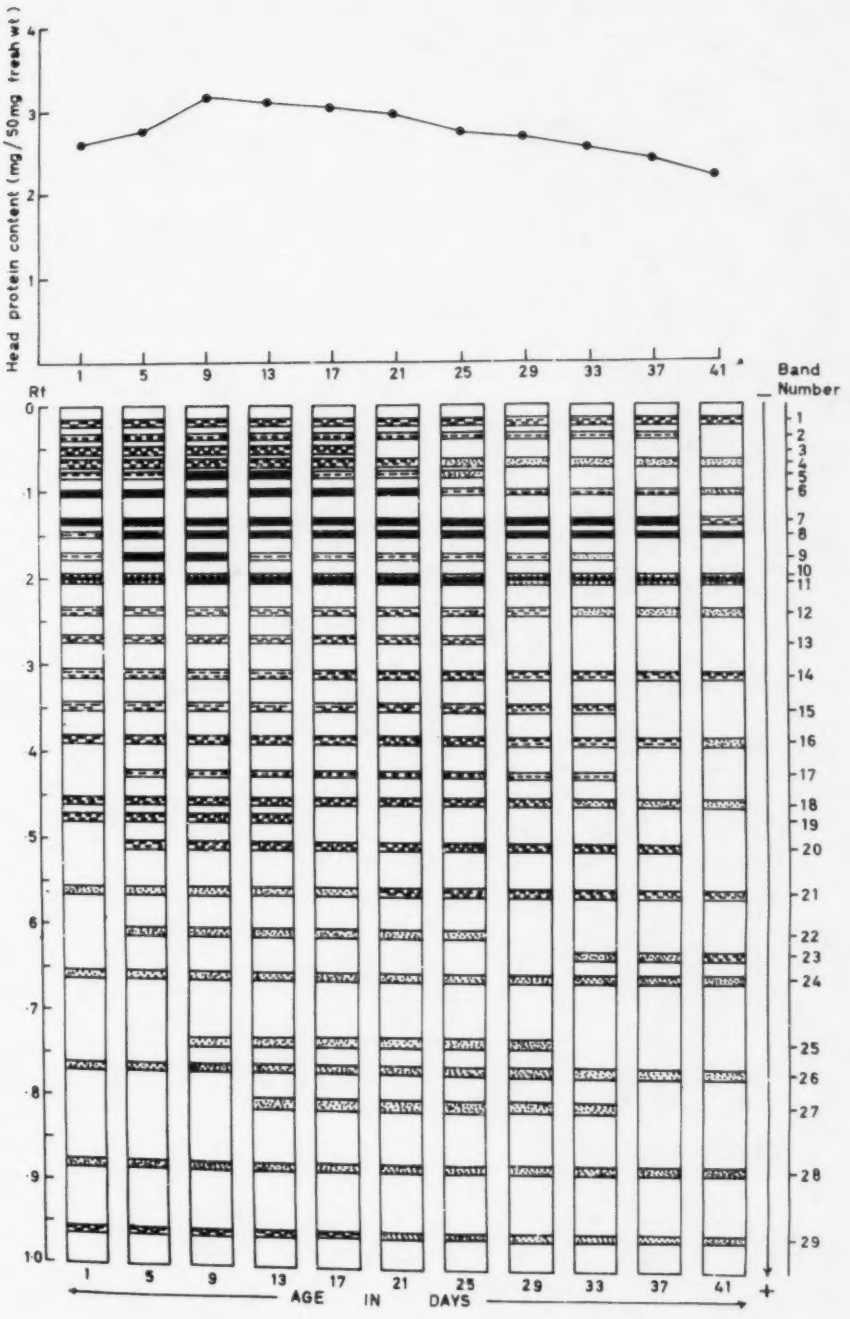

FIG. 1. Head protein content and protein banding pattern of female *Zaprionus paravittiger* during aging. Key to intensity of bands ▬ major ▭ minor ▭ trace.

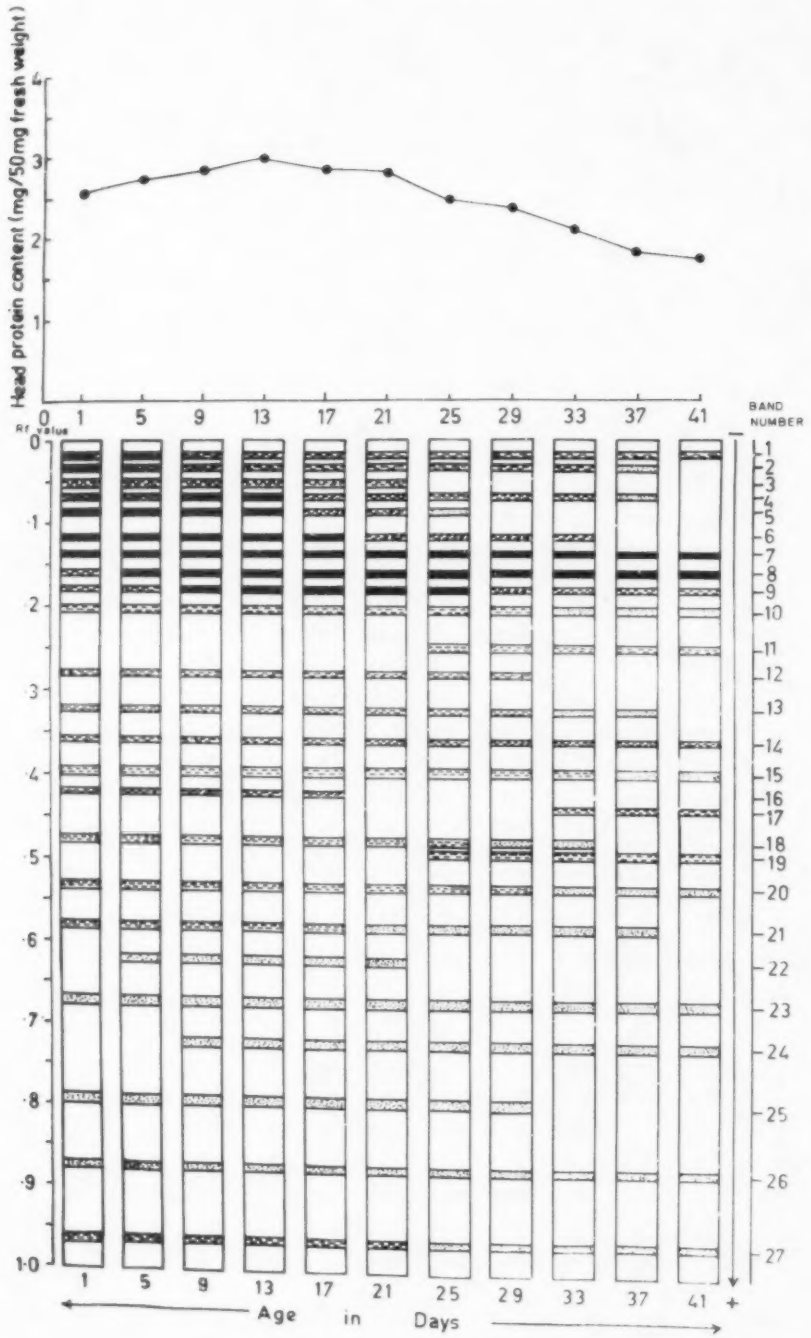

FIG. 2. Protein content and protein banding pattern of heads of male *Zaprionus paravittiger* during aging key to the intensity of bands major ▬ minor ▭ trace ▭

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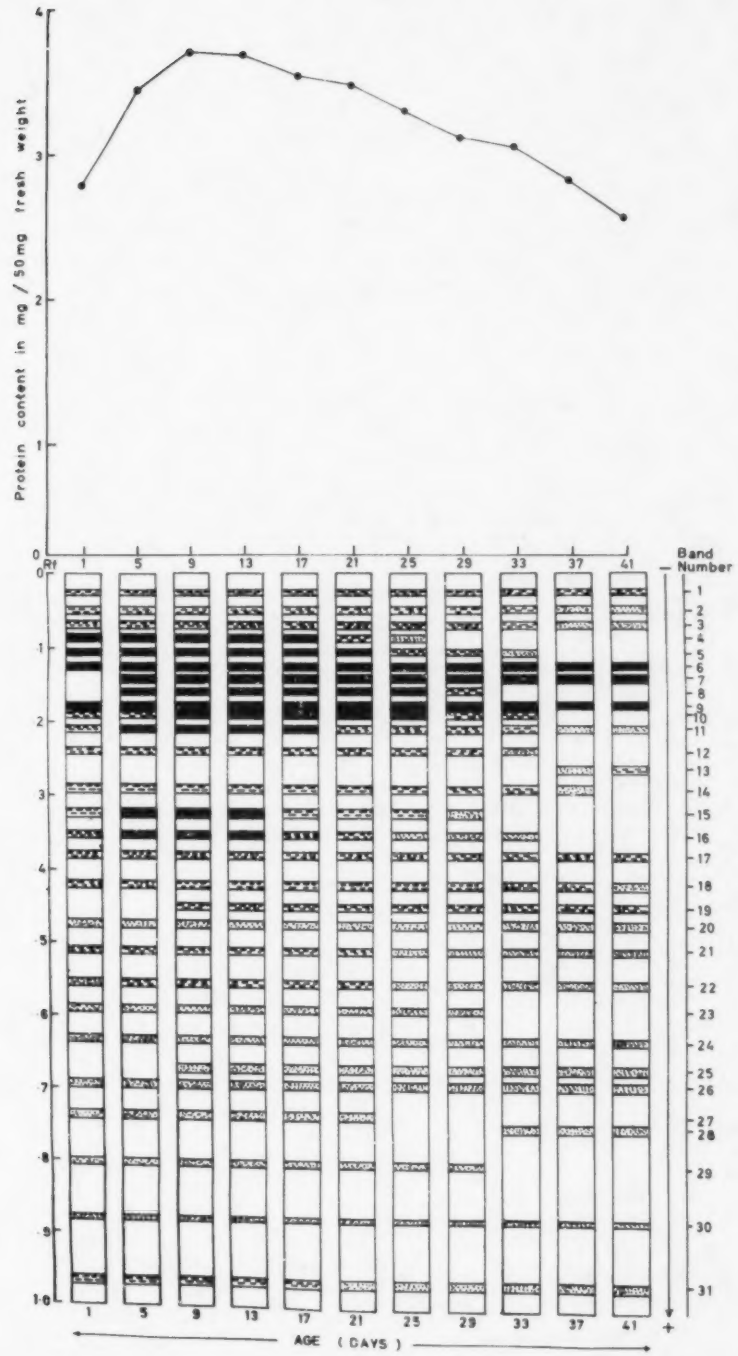

FIG. 3. Protein content and electrophoretic banding pattern of body proteins of aging female *Zaprionus paravittiger*. Key to intensity ▬ major ▭ minor trace ▬

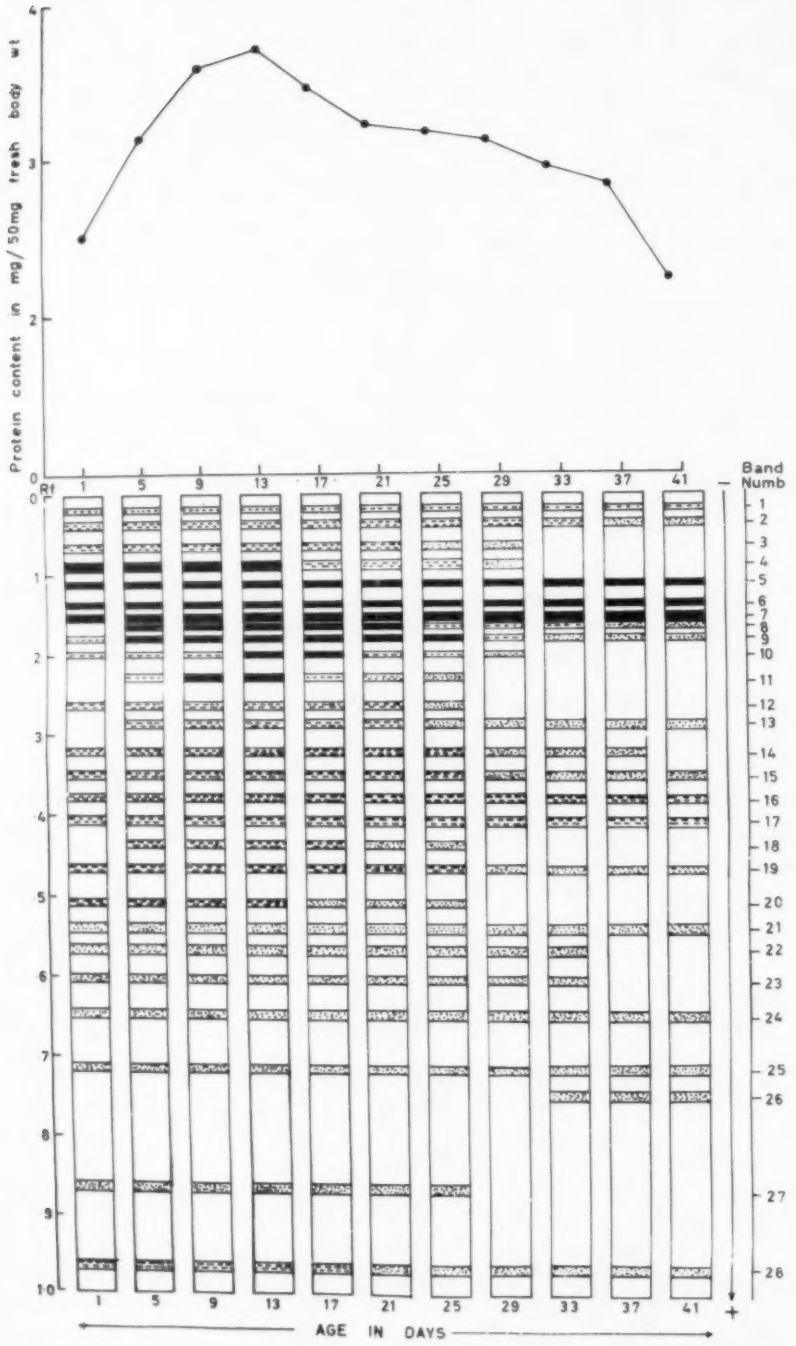

FIG. 4. Protein content and electrophoretic banding pattern of body protein of male *Z. paravittiger*.

TABLE 1. BANDING PATTERN OF HEAD PROTEINS OF AGING FEMALE *Z. PARAVITTIGER*

S.No.	Age (days)	Total number of bands	Number of bands			Missing*	New*
			Major	Minor	Trace		
1.	1	23	2	17	4	–	–
2.	5	26	4	17	5	–	17,20,22
3.	9	27	6	15	6	–	17,20,22,25
4.	13	28	5	16	7	–	17,20,22,25,27
5.	17	27	4	16	7	19	17,20,22,25,27
6.	21	26	4	15	7	19,3	17,20,22,25,27
7.	25	26	3	14	9	19,3	17,20,22,25,27
8.	29	23	2	14	7	19,3,5,13	17,20,25,27
9.	33	23	2	11	10	19,3,5,13	17,20,27,23
10.	37	19	2	9	8	19,3,5,13,9,15	20,23
11.	41	17	1	7	7	19,3,5,13,9,15,2	23

*Compared with one day-old flies

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old bodies and from 3.060 ± 0.079 and 3.045 ± 0.035 in 13 day-old insects heads to 2.019 ± 0.038 and 1.982 ± 0.022 in 41 day-old heads of female and male insects respectively. Such decrease in protein contents is statistically very significant ($p < 0.001$). Thus the quantitative changes were reflected during qualitative expressions. The changes observed in proteins during aging might cause decline in adaptability of insects even to normal environment during old age. The present work is in agreement with the work of Utsami and Natori (1980). The observations on protein changes during aging of *Z. paravittiger* are suggestive of selective expression and repression of genes and support the gene regulation theory of aging proposed by Kanungo (1970, 1975, 1980).

In conclusion, it can be said that aging is accompanied by increase and decrease of selective proteins. But the overall protein content declines with increasing age. These changes lead the organism towards physiological decline and also reduce the adaptability of the organism towards its environment with advancing age.

TABLE 2. BANDING PATTERN OF HEAD PROTEINS OF AGING MALE *Z. PARAVITTIGER*

S.No.	Age (days)	Total number of bands	Number of bands			Missing*	New*
			Major	Minor	Trace		
1	1(0–24 hr)	22	3	16	3	–	–
2	5	23	4	15	4	–	22
3	9	24	6	13	5	–	22,24
4	13	24	6	14	4	–	22,24
5	17	24	4	15	5	–	22,24
6	21	23	3	14	6	16	22,24
7	25	23	3	13	7	16,3	24,11,19
8	29	22	2	13	7	16,3,5	24,11,19
9	33	21	2	10	9	16,3,5,12,25	24,11,19.17
10	37	19	2	6	11	16,3,5,12,25,6,18	24,11,19,17
11	41	15	2	6	7	16,3,5,12,25,6,18 2,4,13,21	24,11,19,17

*Compared with one day-old flies

TABLE 3. ELECTROPHORETIC BANDING PATTERN OF BODY PROTEINS OF AGING FEMALE *Z. PARAVITTIGER*

S.No.	Age (days)	Total number of bands	Number of bands			Missing*	New*
			Major	Minor	Trace		
1	1(0–24 hr)	25	4	13	8	–	–
2	5	27	9	11	7	–	7,8
3	9	29	10	11	8	–	7,8,19,25
4	13	29	10	11	8	–	7,8,19,25
5	17	29	8	13	8	–	7,8,19,25
6	21	29	6	14	9	–	7,8,19,25
7	25	28	5	11	12	27	7,8,19,25
8	29	27	3	12	12	27,4	7,8,19,25
9	33	24	3	9	12	27,4,15,23	7,19,25,28
10	37	21	3	4	14	27,4,15,23,5,12,16	7,19,25,28,13
11	41	20	3	4	13	27,4,15,23,5,12,16,14	7,19,25,28,13

*Compared with one day-old flies

TABLE 4. ELECTROPHORETIC BANDING PATTERN OF BODY PROTEINS OF AGING MALE *Z. PARAVITTIGER*

S.No.	Age (days)	Total number of bands	Number of bands			Missing*	New*
			Major	Minor	Trace		
1	1(24-hr)	23	4	13	6	–	–
2	5	27	6	15	6	–	8,11,13,18
3	9	27	7	14	6	–	8,11,13,18
4	13	27	8	13	6	–	8,11,13,18
5	17	27	6	14	7	–	8,11,13,18
6	21	27	5	13	9	–	8,11,13,18
7	25	27	4	10	13	–	8,11,13,18
8	29	22	3	6	13	12,20,27	8,13
9	33	20	3	5	12	12,20,27,10,3,4	8,13,26
10	37	18	3	3	12	12,20,27,10,3,4,22,23	8,13,26
11	41	17	3	3	11	12,20,27,10,3,4,22,23,14	8,13,26

*Compared with one day-old flies

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THE INFLUENCE OF INSULIN IN THE CELL CYCLE OF HUMAN AGING CELLS

JUDITH L. CHIGER and HIDEKO KAJI

Department of Pharmacology Jefferson Medical College 1020 Locust Street Philadelphia, PA 19107

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INTRODUCTION

IT IS widely accepted that with aging there is an altered responsiveness of tissues to hormones (Shock, 1977). This may be a result of changes in receptor concentration, or in the cell's sensitivity to the hormone (Roth, 1980). A deterioration of glucose tolerance and an increasing insensitivity to the anabolic hormone insulin is also seen with senescence *in vivo* (Shock, 1977; Roth, 1979). This age-related alteration in responsiveness has been reported *in vitro* for some of the metabolic actions of insulin, including glucose uptake and ^{14}C-uridine incorporation (Fujimoto and Williams, 1922). Insulin is capable of eliciting other effects, such as alterations in the membrane transport of ions and amino acids, regulation of enzymatic activities, and stimulation of protein and DNA syntheses (Krahl, 1972; Czech, 1977; Hilf *et al.*, 1981), both *in vivo* and *in vitro;* however, alterations with senescence have not been reported for all the actions of this hormone.

It is well established that human diploid fibroblasts *in vitro* have a finite life span, undergoing a period of rapid proliferation, followed by a period of declining ability to initiate DNA synthesis and a decreased growth rate, leading to cell death (Hayflick, 1965). For this reason the use of these cells in culture is a convenient means to study the effects of insulin at macromolecular and cellular levels with respect to aging.

The studies presented here were undertaken to examine the effect of insulin, at physiologic doses, on DNA synthesis in human diploid fibroblasts, and to observe any age related differences in response to the hormone that might occur.

MATERIALS AND METHODS

Studies of insulin effect on DNA synthesis

IMR 90 cells (Nichols *et al.*, 1977) or their SV40 virus transformant (AG2804) were grown in a Linbro 24-well dish. Before approaching confluence, cell monolayers in each well were washed twice with MEM without fetal

All correspondence: Dr. Hideko Kaji, Thomas Jefferson University Department of Pharmacology 1020 Locust Street Philadelphia, PA 19107

calf serum and then incubated with MEM without fetal calf serum, but containing 0.01% bovine serum albumin. Various concentrations of insulin (kindly supplied by Eli Lilly Co.) were also added at this time to appropriate test cultures. These concentrations are given in the results and figure legends. After 48–60 hours incubation in the absence of fetal calf serum, the MEM was removed and the cells were washed once with MEM. The cells were then fed with one ml MEM containing 5% fetal calf serum to initiate the cell cycle.

At various hours after the cell cycles were initiated, wells were washed twice with MEM and 0.5 ml of MEM containing 0.5 μCi ^{3}H-thymidine (48 Ci/mmol) was added. The plate was further incubated in an atmosphere of 95% air and 5% CO_2 at 36°C for one hour. After incubation the cells were washed three times with phosphate buffered saline-PBS (7.650 g NaCl/0.725 g Na_2HPO_4/0.212 g KH_4PO_4 per liter) and dissolved in 0.2 ml of 0.3% SDS. For measurement of ^{3}H-thymidine incorporation an aliquot of 0.5 ml was processed in trichloroacetic acid for insoluble radioactivity measurement at 0°C (Mans and Novelli, 1961) and counted in a liquid scintillation counter. An aliquot of 0.1 ml was used to determine protein concentration by the method of Lowry (Lowry *et al.*, 1951). Determination of cell number was carried out using a hematocytometer.

Autoradiography

Various fibroblasts were seeded at a density of 1.0 ~ 1.4 × 10^4 cells/ml in 60 mm Petri dishes containing 6 (22 × 22 mm) glass coverslips, grown, fasted, and processed for thymidine incorporation as described before except that the amount of ^{3}H-thymidine is 0.1 μc/ml.

At appropriate intervals, coverslips were removed from the Petri dishes, rinsed three times in PBS and fixed in Carnoy's solution (absolute ethyl alcohol:chloroform:glacial acetic acid, 60:30:10). This was followed by a hydration through an alcohol series and air drying overnight. The coverslips were then fixed to glass microslides (3 × 1 inch) and dipped in emulsion (Kodak NTB-2). Following the exposure period (4 days), the slides were developed (Kodak D-19 developer, 5 minutes), fixed (Kodak acid fixer, 5 minutes), and rinsed in deionized water. The cells were then stained lightly with Harris hematoxylin, counterstained with 0.5% aqueous eosin, air dried, counted (500 cells) per covership for labeled nuclei (5 silver grains or more) and all coverslips were prepared in duplicate.

Statistical methods

To test significance, p values were calculated using a two-tailed Student t-test. All numbers in tables present the mean experimental values ± one standard deviation.

RESULTS

Cultures of IMR90 and AG2804 were treated as described in Materials and Methods. As can be seen from Figure 1, cells not exposed to insulin showed a minimal level of ^{3}H-thymidine incorporation prior to addition of serum and a lag period of 12 to 14 hours before incorporation and DNA synthesis were increased. A peak was reached in early passage cells at 22 hours and middle and late passage cells at 24 hours after initiation of the cell cycle. The transformed cells, AG2804, were poorly synchronized but appeared to reach a broad peak incorporation of ^{3}H-thymidine 14 to 26 hours after initiation of the cell cycle. Cells lose their dependence on growth factors as a result of transformation, and this loss of dependence makes it difficult to synchronize these cells through serum deprivation.

As the cells aged, a delayed entrance into the S phase was observed. Late passage cells expressed a longer lag period prior to DNA synthesis than either early or middle passage cells. The peak incorporation of ^{3}H-thymidine was also much lower for late passage or older cells than for the younger fibroblasts as has been reported elsewhere (Pledger *et al.*, 1979; Chiger *et al.*, 1982).

When these cultures were exposed to various doses of insulin during the serum deprivation or "fasting" period, there appeared to be no effect on the synchronization of these cultures (Figure 1). As with the untreated cells, there was a mimimal level of ^{3}H-thymidine incorporation prior to reinitiation of the cell cycle followed by a lag period and entrance

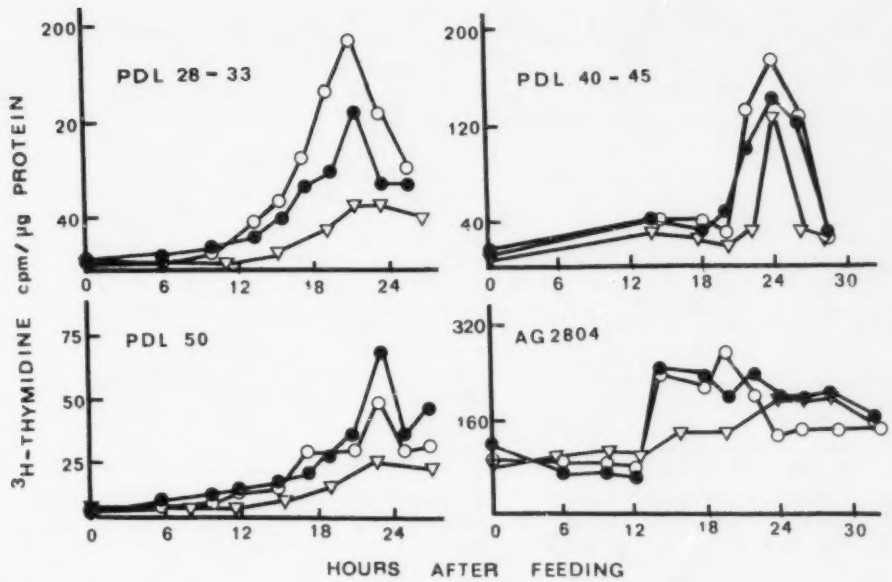

FIG. 1. Effect of insulin on the incorporation of ^{3}H-thymidine in normal and SV-40 virus transformed fibroblasts. Cultures were synchronized and treated as described in Materials and Methods. (a) PDL 28–33; (b) PDL 40–45; (c) PDL 50; (d) SV40 virus transformed (AG2804), Insulin concentrations tested: 5 ng/ml (○), 6,000 ng/ml (▽), control (●).

into the S phase and DNA synthesis. This was consistent with results reported elsewhere (Pledger *et al.*, 1979; Paul *et al.*, 1978).

The presence of insulin during the "fasting" period did, however, alter the incorporation of ^{3}H-thymidine. When exposed to physiologic concentrations of insulin (5 ng/ml), early passage cells showed an increased incorporation of ^{3}H-thymidine into cold TCA precipitable material (Figure 1, Table 1). This increase was dose dependent and was observed with concentrations ranging from 5 ng/ml to 100 ng/ml. Middle passage cells (PDL 40–45) also showed an increased incorporation of ^{3}H-thymidine, but within a narrower range of concentrations (10 ng/ml to 50 ng/ml). Late passage cells (Figure 1) showed no significant change in DNA synthesis within these concentration ranges. Thus, there appears to be an age-dependent sensitivity of these fibroblasts to insulin's stimulatory effect on DNA synthesis.

AG2804 cells (Figure 1) showed no significant changes in ^{3}H-thymidine incorporation into DNA within the lower concentration range tested (10 ng/ml to 100 ng/ml).

When the cell cultures were exposed to supraphysiologic concentrations of insulin, all cells studied were affected similarly, showing a decreased incorporation of ^{3}H-thymidine into DNA. Although both early and late passage cells were affected with a reduction in DNA synthesis of approximately 40 percent when exposed to 6,000 ng insulin/ml, middle and late passage cells were more sensitive, expressing this decrease at 100 ng/ml. AG2804

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TABLE 1. EFFECT OF INSULIN ON INCORPORATION OF ^{3}H-THYMIDINE

	Doses (ng/ml)						
	5	*10*	*30*	*50*	*100*	*1,000*	*6,000*
	% of Control						
PDL 28–33	158.7[d] ±20.7	139.8[a] ±18.4	132.4 ±25.2	142.7[a] ±6.2	131.5[b] ±17.8	57.3 ±27.5	59.1[a] ±10.16
PDL 40–45	105.7[c] ±4.2	124.3 ±18.3	133.4 ±33.7	126.0 ±11.2	87.4[b] ±7.2		
PDL 50		86.6 ±21.1	95.7 ±9.4	88.8 ±28.7	65.6[b] ±3.0	76.4 ±9.7	63.3[d] ±17.5
AG2804		100.0 ±4.9	96.9 ±0.1		124.9 ±15.3		88.2[d] ±2.3

Experimental conditions are described in Materials and Methods. Four points during peak DNA synthesis (S phase) were used as the sum of radioactivity incorporated into cold TCA precipitable material per ug protein.
[a]$p < 0.001$; [b]$p < 0.01$ when compared with control
[c]$p < 0.02$; [d]$p < 0.05$ when compared with control

cells also showed a significant decrease in DNA synthesis, but to a lesser extent than normal fibroblasts.

Autoradiographic studies were undertaken in order to determine the percentage of the population of cells that was entering the S phase. The results of these experiments are shown in Table 2. IMR90 fibroblasts, PDL 28–33, treated with 10 ng insulin/ml during the quiescent period exhibited an increase in percent labeled nuclei over control cultures as early as 12 hours after initiation of the cell cycle and this increase continued throughout. IMR90 fibroblasts at PDL 40–50 and AG2804 cells exhibited no significant alterations in the percent labeled nuclei between control and insulin treated cultures. Treatment of early passage IMR90 fibroblasts with 6,000 ng insulin/ml decreased the number of cells entering the cell cycle. Autoradiographic studies showed that only 72.6 percent of the treated cells entered S phase as compared with 80.8 percent of control cells. This may explain the decreased ^{3}H-thymidine uptake seen in IMR90 fibroblasts exposed to 6,000 ng insulin/ml. In addition, it was observed that a lower percentage of senescent IMR90 fibroblasts (PDL 50) enter the S phase than young IMR90 fibroblasts (PDL 28–33) (Table 2). The entrance into the S phase of the cell cycle of PDL 50 IMR90 fibroblasts was delayed corresponding to the longer lag period expressed in the ^{3}H-thymidine uptake studies (Table 1). Together this indicates that the lower peak incorporation seen with older cells was probably due to a decreased number of cells in the culture traversing the cell cycle, as has also been reported elsewhere (Grove and Cristofalo, 1978).

It has been reported that insulin increased incorporation of radioactive amino acids into protein, leading to an increased protein content of cells in culture (Fujimoto and Williams, 1974; Chiger *et al.*, 1982; Rubin, 1976; Griffiths, 1970) (data not shown). Increase in protein content was only seen at the higher dosage and no statistically significant difference were observed in cell counts throughout the cell cycle when cells were treated with insulin.

TABLE 2. EFFECT OF INSULIN ON THE LABELING OF NUCLEI BY ^{3}H-THYMIDINE DURING THE CELL CYCLE

Cell Type	Hours after Initiation of Cell Cycle	% Labeled Nuclei		
		Control	10 ng/ml	6,000 ng/ml
PDL 28–33	1	1.1 ± 0.7 (5)	1.8 ± 0.9 (5)	1.1 ± 0.4 (3)
	12	15.9 ± 5.2 (5)	21.8 ± 2.3 (5)[a]	18.5 ± 3.4 (3)
	18	41.9 ± 10.1 (4)	65.3 ± 2.5 (3)[c]	48.5 ± 4.9 (3)
	21	64.8 ± 2.3 (3)	76.8 ± 3.5 (3)[c]	60.3 ± 1.9 (3)
	24	71.6 ± 10.1 (5)	81.9 ± 2.7 (6)[c]	64.5 ± 1.7 (3)
	27	80.8 ± 2.4 (5)	87.2 ± 2.4 (5)[b]	72.6 ± 2.3 (2)[b]
PDL 40–45	1	1.0 ± 0.5 (4)	1.0 ± 0.3 (4)	
	12	8.7 ± 0.1 (2)	10.2 ± 1.0 (2)	
	14	7.8 ± 1.1 (2)	10.4 ± 0.3 (2)	
	21	20.8 ± 12.4 (2)	29.3 ± 10.5 (2)	
	25	48.7 ± 6.2 (4)	48.7 ± 10.5 (4)	
	28	50.8 ± 1.7 (2)	53.2 ± 3.1 (2)	
PDL 50	1	0.7 ± 0.4 (2)	0.3 ± 0.1 (3)	
	12	3.3 ± 2.2 (3)	3.9 ± 0.7 (3)	
	24	26.5 ± 2.1 (3)	26.7 ± 5.2 (2)	
	26	30.5 ± 1.5 (3)	29.9 ± 10.0 (3)	
AG2804	1	6.0 ± 1.4 (2)	5.9 ± 0.7 (2)	
	12	63.9 ± 9.4 (4)	66.4 ± 5.8 (4)	
	18	72.6 ± 2.1 (2)	71.5 ± 7.8 (2)	
	21	82.6 (1)	72.0 ± 5.1 (2)	
	25	82.7 ± 5.0 (4)	80.6 ± 5.7 (4)	
	28	86.7 ± 2.1 (2)	86.0 ± 1.4 (2)	

Experimental conditions are described in Materials and Methods. Number in parenthesis represents number of samples.

[a]$p < 0.001$; [b]$p < 0.001$; [c]$p < 0.05$, when compared with control

DISCUSSION

When normal human fibroblasts (IMR90) were treated with physiologic concentrations of insulin, an increased incorporation of ^{3}H-thymidine into cold TCA precipitable material was observed upon re-initiation of the cell cycle. This increase was both age and dose dependent. A similar lag phase was seen prior to entrance into S phase and initiation of DNA synthesis in both treated and untreated fibroblasts; however, once DNA synthesis had begun, more ^{3}H-thymidine was incorporated per μg protein in insulin treated cells than in control cells throughout the S phase. This may be indicative of a greater number of cells entering S or an increased rate of DNA synthesis. The data from the autoradiographic studies are suggestive of changes in the fraction of cells entering S phase. IMR90 fibroblasts, PDL 28–33, exposed to 10 ng insulin/ml exhibited a larger fraction of cells entering S phase than untreated cultures. Senescent cells, PDL 40, and SV40 virus transformed cells were insensitive to insulin's stimulatory effects. This decreased sensitivity to insulin with age and transformation was also seen in ^{3}H-thymidine uptake studies. This supports previous observations that aging and transformed cells lose their ability to respond to various growth factors (Paul *et al.*, 1978, Stiles *et al.*, 1979 Pardee *et al.*, 1979).

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Early passage fibroblasts exposed to insulin appeared to enter the S phase faster than untreated cells indicating that insulin may accelerate the progression of the cells through G_1 phase as well as increase the percentage of cells traversing the cell cycle. This has also been hypothesized by Pledger *et al.* Using microfluorometric analysis it was observed that BHK cells treated with insulin exhibited cell cycles skewed toward the S phase. The duration of the S phase, which is reflected by the synthesis of DNA, appears unaffected in these studies.

A series of biochemical reactions that are directly related to the onset of DNA synthesis occur during the lag phase (Rao and Sunkard, 1978). Serum, which can initiate the cell cycle in quiescent cultures, contains various growth factors and hormones. These factors set up the biochemical events of G_1, as well as regulate the rate at which the cell traverses G_1 and enters S (Paul *et al.*, 1978; Sato, 1975; de Asua *et al.*, 1978). Insulin alone is incapable of eliciting this reaction (Paul *et al.*, 1978); however, in concert with other growth factors and hormones insulin can modulate the entrance into the cell cycle (de Asua *et al.*, 1978; Armelin, 1975). Insulin may in some way affect one or more of these events during the quiescent period, "priming" the cells prior to growth factor addition to the media, thus facilitating traverse through G_1 and into S. This modulation of the cell cycle by insulin may also involve the entrance of a greater percentage of quiescent cells into the cell cycle.

A decreasing sensitivity with age to the actions of insulin has been reported in vivo (Shock, 1977; Roth, 1980), as well as *in vitro* (Fujimoto and Williams, 1974). In the data presented here, an age-dependent response to the effects of insulin on ^{3}H-thymidine incorporation is seen. Late passage human fibroblasts, not treated with insulin, traverse the cell cycle poorly as compared with early passage younger cells. In these studies, insulin was incapable of enhancing the progression of these older cells through the cycle. However, it was capable of doing so in the early and middle passage cells, at concentrations within the physiologic range.

AG2804 cells are difficult to synchronize in culture. This has been attributed to a decreased requirement for growth factors in media upon transformation of cells by SV40 virus (Lowry *et al.*, 1951; Stiles *et al.*, 1979; Pardee *et al.*, 1979). This decreased growth factor requirement may account for the results seen here (Figure 1).

Supraphysiologic doses of insulin appeared to prevent or delay the entrance of a population of cells into the cell cycle. This decrease in DNA synthesis was seen in all age groups of cells. Griffiths (1970) reported a depression in DNA synthesis in WI-38 cells exposed to 40 μg insulin/ml. It has been suggested that physiologic concentrations of insulin bind to insulin receptors and supraphysiologic concentrations bind to other receptors for structurally related peptides (Petrides and Bohlem, 1980). This overlap interaction may be responsible for the results seen.

SUMMARY

Normal human IMR 90 fibroblasts at population doubling levels (PDL) 28 ~ 33 had an increased incorporation of ^{3}H-thymidine into DNA when the cells were pretreated with physiologic concentrations of insulin (5 ~ 10 ng/ml) prior to re-initiation of the cell cycle. Insulin stimulation was not observed with fibroblasts at PDL 50 nor with SV40 virus transformed cells. In all cells treated with supra-physiologic concentrations of insulin (1000 ~ 6000 ng/ml) prior to the re-initiation of cell cycle, the incorporation of

thymidine into DNA was greatly diminished. Autoradiographic studies of the effect of insulin suggest that a larger fraction of cells was found to enter S phase than untreated cultures. This effect was evident in younger cells rather than senescent cells.

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LIFESPAN AND PROTEIN SYNTHESIS IN SEVERAL *BOMBYX MORI* MUTANTS WITH GENETIC ABNORMALITIES IN AMINO ACID AND PROTEIN METABOLISM

MINORU OSANAI

Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo, Japan

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Abstract—Three kinds of "white eggs" and red-blooded *Bombyx* mutants (w_1, w_2, w_3 and rb) which lack enzymes in tryptophan metabolism showed almost no difference in their lifespans from that of normal stock. However, other *Bombyx* mutants, such as Nd, Nd-s and DES-Nd, unable to synthesize fibroin, had shorter lifespans than normal. Among these fibroin-deficient mutants, only the lifespan of DES-Nd was similar to the normal. However, further analysis of the amino acid composition in the cocoon of the mutant showed that its lifespan was also dependent on its ability to synthesize fibroin-like protein. It is very likely that, different from abnormalities in amino acid metabolism, a genetic abnormality in protein metabolism affects the maintenance of adult life in silkmoths and this results in the short lifespan.

Generally speaking, an abnormality in amino acid metabolism seems to have no influence on silkmoth lifespan. However, an abnormality in protein metabolism results in shortening lifespan. The greater the abnormality in protein metabolism possessed by a silkmoth, the shorter the lifespan.

INTRODUCTION

THE SILKMOTH *Bombyx mori* represents a closed system, since this insect eats nothing after the larval stage. It maintains life by substances accumulated during the larval stages (Tojo *et al.*, 1980). Because they are a closed system and because of their limited lifespan (Osanai, 1978), silkmoths are good materials for biochemical analysis of the aging of metabolic mechanisms for many substances, especially nitrogen-containing substances such as proteins, amino acids and their catabolic endproducts (Osanai and Kikuta, 1981). The changes in amino acid pool sizes during the silkmoth adult lifetime from emergence to senescence can be divided into three stages (Osanai and Kikuta, 1981), which correspond with the three phases of increasing moth death numbers by age (Osanai, 1978). Three active changes in protein metabolism accompany the three changes in amino acid pool sizes. These metabolic changes were assumed to be caused by the development of programmed gene actions with advanced age. The protein metabolism-dependent lifespans of some silkmoth mutants, in which either amino acid or protein metabolism were genetically blocked by the lack of specific genes, are demonstrated in the present paper.

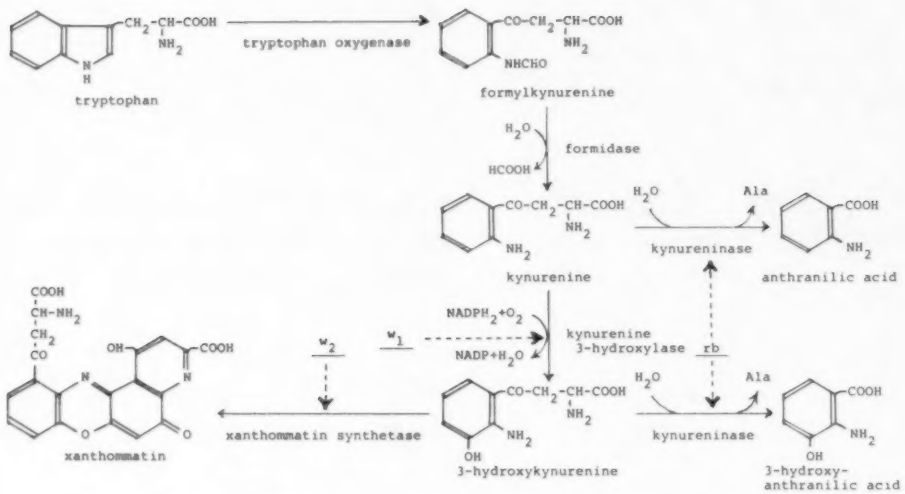

FIG. 1. Tryptophan metabolism in *Bombyx mori*. Broken lines show the genetically blocked cites of reactions in mutants, w_1, w_2 and rb. Enzymatic deficiency of w_3 is not yet known.

MATERIALS AND METHODS

In *Bombyx mori* several genetic mutants (Osanai, 1968) exist which have an abnormality in tryptophan metabolism, namely the white egg and the white eyed silkworms, w_1, w_2 and w_3 and the red-blooded silkworm, rb (Inagami, 1955). White eyed and white egg mutants are not able to synthesize the red pigment, ommochrome, which originates from tryptophan (Koga and Osanai, 1967), because of blocking certain metabolic sites. Another mutant, rb, is kynureninase-deficient, as summarized in Figure 1.

There are also some other silkworm mutants such as Nd (Nakano, 1937), and Nd-s (Horiuchi *et al.*, 1963) which lack the ability to produce the silk protein, fibroin. Another mutant DES-Nd was recently discovered by Dr. T. Gamo in the Sericultural Experimental Station, Tsukuba, Japan. Since the posterior division of their silkgland is genetically degenerated, they synthesize only another component protein, sericin. Among the three mutants, the most strongly inhibited one, Nd (naked) barely spins a cocoon. A large number of mature larvae of the weakly inhibited mutant, DES-Nd, spin a cocoon composed of only sericin, while a few larvae spin no cocoon. Nd-s (sericin-cocoon) are characteristically between other mutants in having moderate cocoon-making ability. As a normal silkworm strain, Daizo was adopted. Their cocoon is colored yellow in the outer layer with fibrous material, but is white in the inner layer. Eggs and larvae of the mutants were kindly supplied by Dr. T. Gamo and Dr. S. Yamamoto, Sericultural Experimental Station, Tsukuba and Kobuchizawa, Japan. The larvae

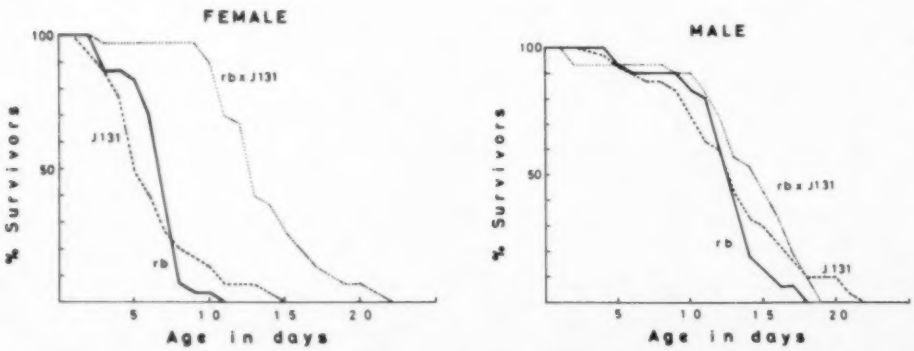

FIG. 2. Survival curves of rb, J131 and their hybrid.

were kindly reared at 25°C by Director M. Kusuno in the Tokyo Metropolitan Sericultural Station, Akigawa. Treatments after pupation were carried out as described in the previous paper (Osanai, 1978).

The Daizo cocoon was divided into three parts, inner and outer layers and cocoon-fibrous material around the cocoon. These three parts and the cocoons of DES-Nd were dissected into very small pieces. About 100 μg of each piece were then hydrolysed in 1 N HCl at 2 atm. for 20 hours, followed by analysis of component amino acids by an amino acid autoanalyser, JLC-6AH (Japan Electron Optic Laboratory) by developing with buffer systems composed of lithium and sodium citrate (Osanai and Kikuta, 1981).

RESULTS

The lifespan of rb adult males is normal and comparable with that of stock silkworm races. However, the lifespan of female rb moths is half that of males (Figure 2). This sexual dimorphism in survival is observed in other silkmoth races and mutants. For example

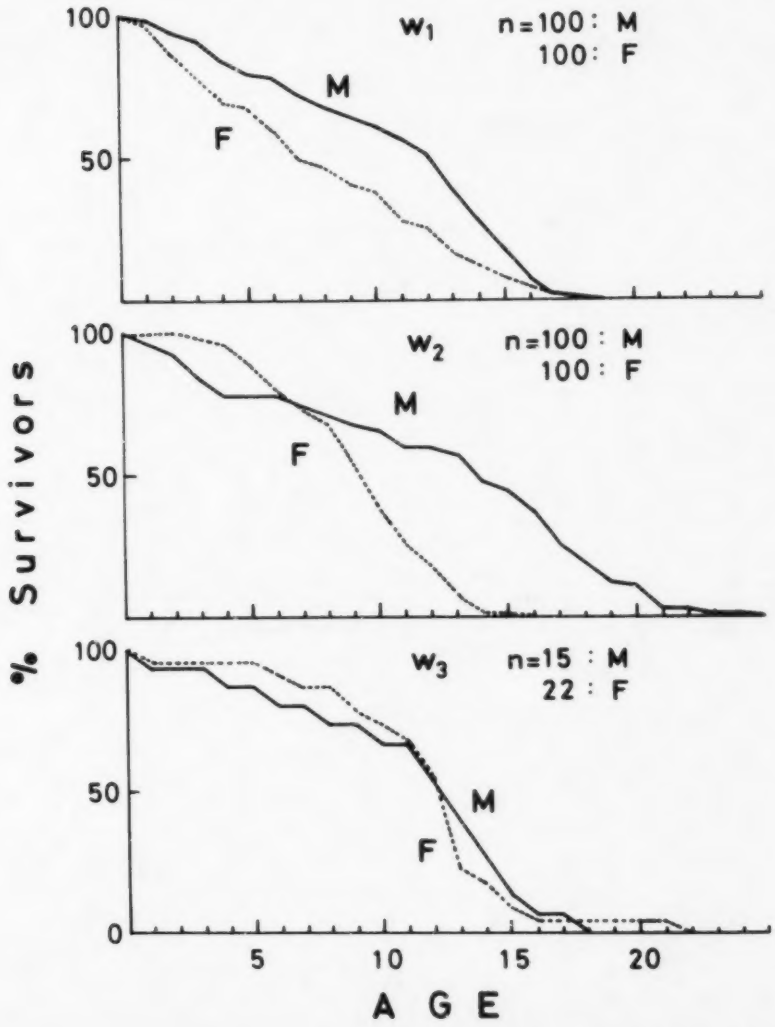

FIG. 3. Survival curves for tryptophan metabolism-blocked, white egg mutants of silkmoth, *Bombyx mori*.

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the normal strain, J131 shows the same pattern, but the female hybrids of the rb and J131 races has a standard lifespan comparable to males. The lifespan of female silkmoth, as a ZW-type animal, may be affected by multiple alleles located on the sex chromosomes.

Three tryptophan metabolism-blocked white-eyed mutants have lifespans similar to that of ordinary stock strains, as shown in Figure 3. Males of all three mutants have normal lifespans. Among the three a sexual difference was not found in the w_3 mutant, but it was noted in the other two white eyed mutants, especially in w_2, which lacks xanthommatinsynthetase. Generally speaking, amino acid metabolism abnormalities seem to have almost no influence on silkmoth lifespan.

Lifespans in silkmoth mutants abnormal in protein metabolism were next measured (Figure 4). Among the three fibroin synthesis blocked mutants, Nd had a marked short

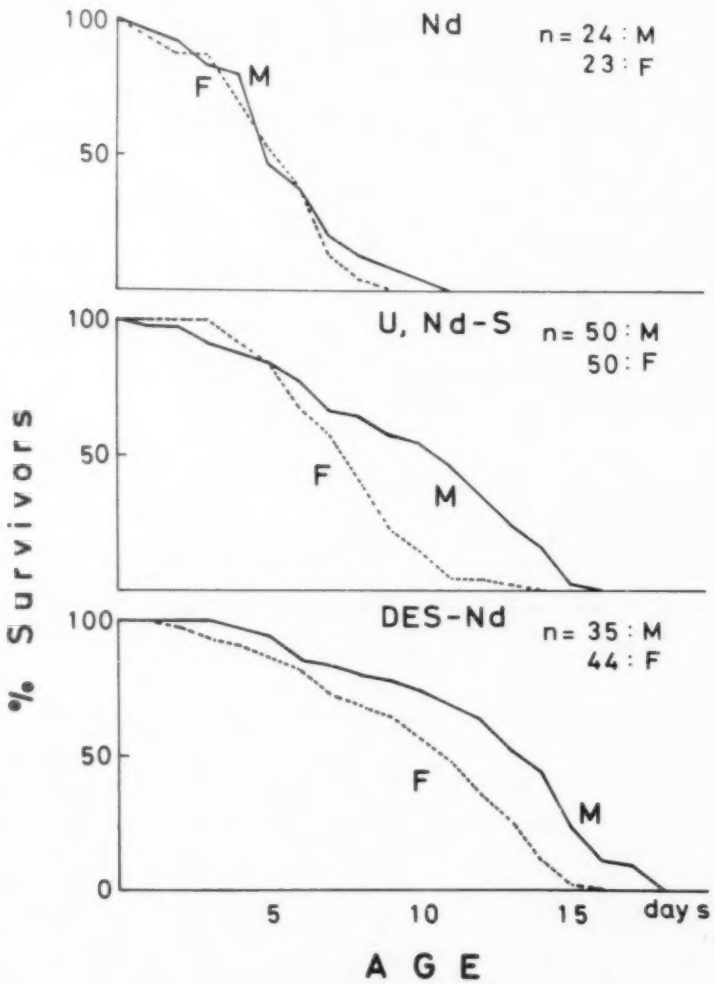

FIG. 4. Survival curves of silkmoth mutants with no ability to synthesize fibroin, Nd, Nd-s and DES-Nd.

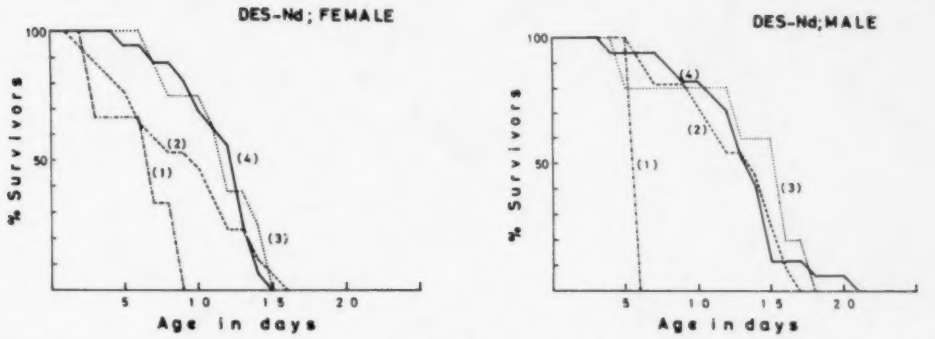

FIG. 5. Survival curves of four subgroups of a weakly affected, fibroin synthesizing blocked mutant, DES-Nd.

lifespans for both sexes. The lifespans for the other two mutants were slightly shorter than the normal in the order Nd-s followed by DES-Nd. The females tended to have shorter lifespans than the males.

The DES-Nd mutant which has, among the three mutants, an almost normal lifespan, was further divided into four groups according to the morphological features of their cocoons: Group 1 made no cocoon, Group 2 made thin and breakable cocoons, Group 3

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TABLE 1. AMINO ACID COMPOSITIONS (MOLE %) IN THE FIBRE PROTEIN OF COCOONS OF THREE DIFFERENT PARTS OF DAIZO AND THREE SUBGROUPS OF A FIBROIN-SYNTHESIZING-BLOCKED MUTANT, DES-ND. ABOUT A 0.1 G SMALL PIECE OF THE FIBRE DISSECTED FROM THE COCOON WAS HYDROLYSED IN 1 N HCL AT 2 ATM. FOR 20 HOURS, FOLLOWED BY ANALYSIS OF COMPONENT AMINO ACIDS BY AN AMINO ACID AUTOANALYSER.

		Des-Nd, white cocoon			*DAIZO, yellow cocoon*			
Amino acid	*Sericin**	*grade 2*	*grade 3*	*grade 4*	*fibrous m.*	*inner layer*	*outer layer*	*Fibroin***
Asp + Asn	14.5	13.6	10.6	8.8	5.6	4.6	4.4	1.3
Thr	8.4	9.1	7.3	6.4	3.9	3.4	3.2	0.9
Ser	32.3	30.5	25.2	22.9	18.2	16.6	16.5	12.1
Glu + Gln	4.8	4.7	3.9	3.3	3.0	2.3	1.9	1.0
Pro	0.4	0.7	–	–	–	–	–	0.3
Gly	13.9	17.5	24.5	28.4	34.8	38.9	39.3	44.5
Ala	4.6	7.9	14.0	16.5	21.6	22.5	23.2	29.3
Val	3.2	3.3	3.0	2.9	2.6	2.5	2.5	2.2
Cys	0.3	0.2	0.1	0.1	0.1	–	0.1	0.2
Met	0.1	–	–	–	–	–	–	0.1
Ileu	0.7	0.8	0.7	0.7	0.7	0.7	0.6	0.7
Leu	1.2	1.2	1.1	1.0	0.8	0.7	0.7	0.5
Tyr	2.6	3.1	3.6	3.8	4.3	4.7	4.9	5.2
Phe	0.4	0.6	0.6	0.7	0.8	0.7	0.8	0.6
Lys	8.4	2.8	2.1	1.7	1.4	0.8	0.7	0.3
His	1.6	1.1	0.8	0.7	0.6	0.4	0.4	0.2
Arg	2.3	2.8	2.4	2.0	1.6	1.2	0.9	0.5
Total	99.7	99.9	99.9	99.9	100.0	100.0	100.1	99.9

*Gamo, Inokuchi and Laufer (1977), originated in average of three data by Dhavakikar (1962), Kirimura (1962) and Lucas and Rudall (1968).

**Lucas and Rudall (1968).

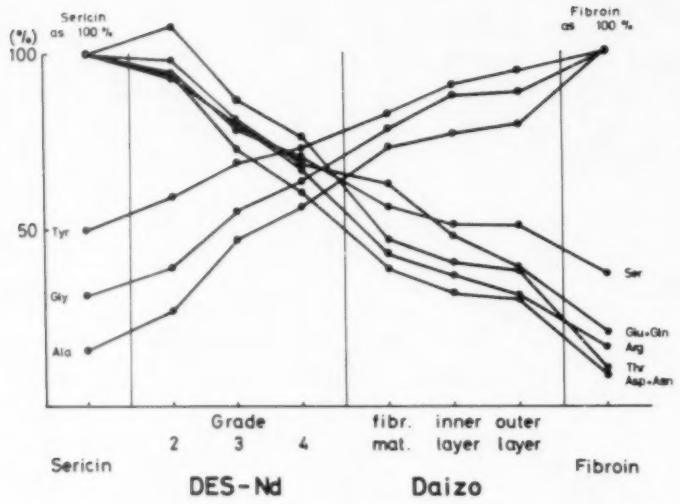

FIG. 6. Percent partition of component amino acids in the fibre protein of cocoons of three DES-Nd subgroups and Daizo. A cocoon of Daizo was divided to three different parts, inner layer, outer layer and fibrous material around cocoon. Five major amino acids composing sericin, Ser, Arg, Thr, Glu + Gln and Asp + Asn and three major amino acids composing fibroin, Tyr, Gly and Ala were adjusted to 100% in each protein, sericin or fibroin, respectively.

made thicker cocoons which were still breakable, while Group 4 made normal appearing cocoons which were difficult to break.

Survival curves for the four subgroups of DES-Nd adults are shown in Figure 5. For males, group 1 adults have a very short lifespan, while the other three groups show lifespans similar to those of ordinary silkmoths. For females, group 1 had the shortest lifespan as observed in males of group 1. The other three female groups had lifespans slightly shorter than those of normal silkmoths with group 2 having the shortest of the three.

The percent of component amino acids in hydrolysed cocoon protein of the three mutant strains was calculated from the results obtained by quantitative determination of a tiny part of the hydrolysed cocoon of each group. These values were compared with those of the inner and outer layers of the cocoon, with the fibrous material of an ordinary silkworm, Daizo, and with those of purified sericin and fibroin (Table 1). Cocoon proteins of each DES-Nd group were chemically nearer to sericin than fibroin, although there were some differences in each group. Even the protein of group 4 cocoons, which are morphologically similar to ordinary cocoon, was very different from not only fibroin but also ordinary cocoon protein.

The same results are more clearly summarized in Figure 6. The major component amino acids of sericin, such as Ser, Arg, Thr, Glu + Gln and Asp + Asn, were adjusted to 100% of sericin and those of fibroin, such as Tyr, Gly and Ala, were adjusted to 100% of fibroin.

DISCUSSION

There is no doubt that protein metabolism has a tight connection with the longevity of animals, since it is controlled by gene actions (Young, 1976). In collagen, α-subunits

decrease with advanced age in spite of increased β-components (Rao *et al.*, 1982). Total plasma protein as well as globulin concentrations were higher in older ewe than in younger, while albumin concentrations were comparable (Koenig and Boling, 1980). The rise in plasma amino acid levels during aging may also suggest a disorder in protein metabolism during human senescence (Armstrong and Stave, 1973). However, the relationship between protein metabolism and the aging process is still unclear for there are a number of conflicting reports. For example Du *et al.* (1977) reported that the synthesis of serum albumin was observed in old mice, whereas there was a lower albumin concentration in the mitochondria. Winterer *et al.* (1976) found that there was no significant difference in protein turnover between young and old animals. A very attractive hypothesis stated by Orgel (1970), the protein error hypothesis of aging was not confirmed in *Drosophila* (Bozcuk, 1976).

The longevity of mammals is strongly affected by the quality and quantity of fed proteins through protein metabolism (Ross, 1972; Miller and Payne, 1968). This has also been confirmed in varied fly species (Tribe, 1966; Gray and Berberian, 1971; Collett, 1976). A fine balance and regulation of protein metabolism appear to be required to maintain animal life.

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The silkmoth is biochemically a semi-closed system, since it takes no food after emergence. The whole adult life is maintained only by metabolism of substances taken and accumulated in the larval stages (Figure 7). Therefore, the lifespan of the silkmoth is naturally limited, although this insect exercises, mates and lays egg as do open system

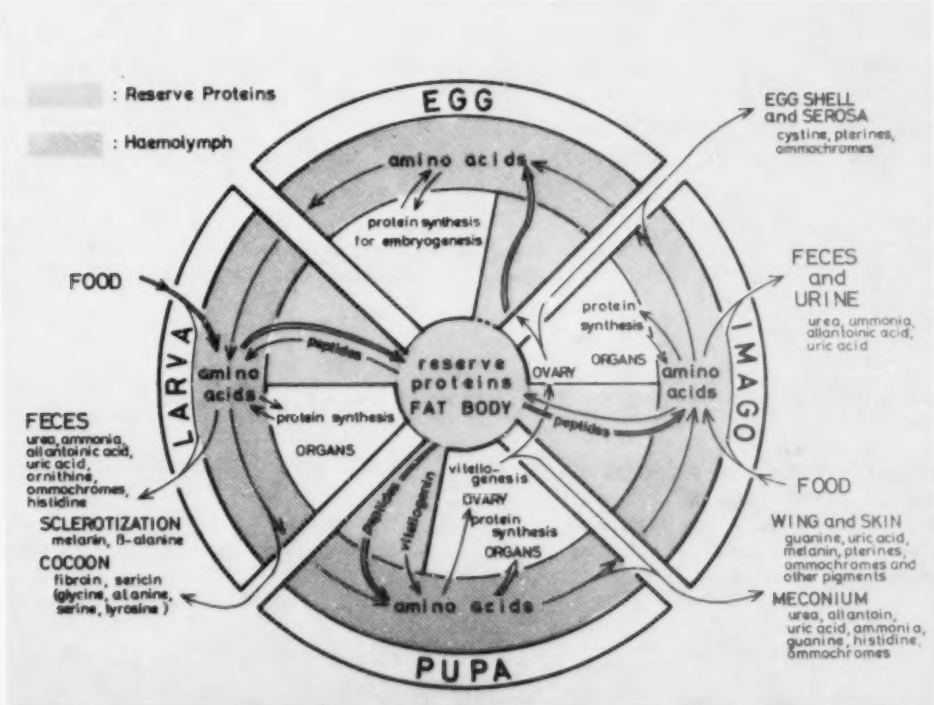

FIG. 7. Interaction of amino acid pools and reserve proteins in whole lifetime of insects. Egg and pupal and adult periods of the silkmoth are closed systems.

animals (Osanai, 1978). The silkmoth is therefore a suitable animal to use for analysis of the relationship between longevity and protein metabolism (Osanai, 1981). In this paper, an analysis of this relationship was examined by use of genetically abnormal mutants of both amino acid and protein metabolism. The silkmoth has another feature which suits it for use as a model animal for these studies. The larvae, whether of a stock or a mutant strain, eat only the mulberry leaf as their sole food. The influences of various sorts of diet (Miller and Payne, 1968; Gray and Berberian, 1971), especially protein-rich or not, are thus omitted.

In a broad view, amino acid metabolism may be regarded as a terminal portion of protein metabolism. While an abnormality in tryptophan metabolism did not affect the lifespan of the silkmoth, an abnormality in the synthesis of the protein fibroin caused a shorter lifespan. Moreover, the shortening of lifespan paralleled the intensity of abnormality in fibroin forming ability. It is, therefore, concluded that an abnormality in amino acid metabolism, which belongs to the terminal catabolism of a big protein, does not fundamentally influence the maintenance of life.

The lack of ability to synthesize fibroin appears to cause not only abnormal features related to fibroin synthesis, but it also influences other parts of protein metabolism including amino acid metabolism. These distortions in both regulation and balance of whole protein metabolism results in the shortening of lifespan. In the Nd mutant, which is inhibited most strongly in its fibroin-forming ability as compared to the used mutants, extraordinary big pupae were often observed. Many of them failed to emerge and these died usually during the pupal stages. The rest of them barely succeed in emergence, but then die immediately. If the silkmoth were a normal strain, it would secret various kinds of amino acids in the form of fibroin at cocoon formation. This is, however, impossible for the Nd-mutant, which is unable to synthesize the protein and utilize the amino acids accumulated in the body. The various amino acids, which should be secreted but are instead accumulated in the big pupa without conversion to fibroin, must cause the abnormality in the regulation of protein metabolism, and then the short lifespan.

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MOTOR PERFORMANCE ACHIEVEMENTS IN RATS OF DIFFERENT AGES

B. JÄNICKE, G. SCHULZE, H. COPER

Freie Universität Berlin Institut für Neuropsychopharmakologie Ulmenallee 30 1000 Berlin 19, BRD

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Abstract—Motor performance of rats of different ages was determined in a cross sectional study. The design includes a test battery of seven motor tasks of graduated complexity. The results show a hierarchical order of impairments of motor functions in aging rats; that is, the more complex the task requirement, the earlier and more pronounced is the susceptibility to deterioration of motor coordination. In spontaneous activity and swimming no difference could be observed between young and old rats. With increasing requirements for the tilting-plane, horizontal wire, climbing and chimney tests as well as the rotarod test, the older rats show a differentiated decrease in performance. The findings are discussed in respect to the theory that aging is a reverse process of early development.

INTRODUCTION

IDENTIFYING AN animal as being chronologically old can be done quite well by observing its motility. In comparison to young and adult individuals, the motor activity of older animals changes both qualitatively and quantitatively. Numerous experiments performed on both laboratory animals and on humans have attempted to assess these changes quantitatively and to describe the process behind them. The analysis was carried out at a variety of levels of organisation and numerous functional relationships (Strehler, 1960; Verzar, 1963; Hahn, 1979; Shock, 1970; Potvin *et al.*, 1980). Accordingly, aging is a dynamic process which starts to develop in adulthood. It is characterized by the fact that in the third phase of life, the organism can increasingly react less adequately to endogenous and exogenous stimuli, that is, its adaptation abilities decrease. This theory has been well supported by studies of reactions to stressful events of the endocrine system, physiological functions (temperature regulation, nourishment in-take), etc. The conditions and achievement demands under which individuals of various ages show a changed motor activity behavior have only rarely been studied. This question will be addressed in this paper.

In order to test the motor performance, test tasks with different levels of complexity of the demands on the course of movement had to be performed on individuals of different ages. Only by using this method could the changed performance capacity in old age be presented and summarized in a cross-sectional analysis. The following motor test batteries were used for testing the performances of aging rats: 1) spontaneous activity; 2) swimming test; 3) tilting plane; 4) horizontal wire; 5) chimney test; 6) climbing test; 7) rotarod test.

A prerequisite for each study of age dependent changes in laboratory animals is to determine to what extent the individuals are "old." For determining this criterion Hahn (1979) postulates a stable survival curve of the collective as well as the development of a Gompertz curve. This curve yields an age dependent death rate and is linear. Further, one needs to define when a research animal can be designated as "old." The adult phase of life begins with puberty. The advancement into the third phase of life, however, cannot be so clearly determined since characteristic changes on the various organisation levels do not occur simultaneously in terms of time and quantity. In addition, there is considerable individual variation in the level of performance. Therefore, the pragmatic 50% survival differentiation has been chosen for characterizing an animal as old. This half-life of a population signifies that point at which half of the original individuals have died. This cannot be compared to the biological age which can only be obtained through a multiple regression analysis (Furukawa *et al.*, 1975; Ingram *et al.*, 1982).

METHODS

Female Wistar rats between the ages of 4–32 months were used for the experiment. The animals were weighed weekly and the death rates were recorded for a period of three years. After the age of 12 months the adult animals were pregnant between four and five times. Up to seven rats were simultaneously housed in (Makrolon) cages (55 × 33 × 20 cm). They were fed (Altromin R10 Fa. Altrogge, Lage/Lippe) and received water *ad libitum,* were maintained in a 12:12 light:dark cycle at a constant room temperature of 21–22°C.

1) Spontaneous activity

After being given time to adapt to their environment, the spontaneous activity of rats of eight different age groups was measured over a period of 72 hours. During these three days, the rats were maintained undisturbed in the laboratory; food and water intake were *ad libitum.* Seven animals each per age group 4, 12, 14, 18, 20, 24, 27, 31 months were evaluated. Each animal was placed on a motilimeter which works under the principle of change in capacity and continually registers the spontaneous activity of the individual rat in its cage. Thus, all motility greater than, for example, chewing movements were recorded.

For ease of computations the recorded impulses were summed up to 30 minute intervals. This data was then put onto a papertape punch for later analysis and stored on discs for a Microprocessor SWTPC 6800. Final analyses were completed with a BASIC program. The spontaneous activity of three consecutive 12 hour dark phases was evaluated and the means of the age groups were compared.

2) Swimming test

The swimming tank used for this test measured 30 × 40 cm with a water depth of 60 cm. Under these conditions a rat, in a vertical position, could barely touch the bottom of the tank with its tail. The water temperature was maintained at 23°C. Here the swimming behavior of the rats was observed using criteria developed by Marshall *et al.* (1979). Accordingly the intensity (vigor) of the swimming movements and the animals' success in maintaining their heads above water for a 15 minute period were registered using a graduated scale. The rats' body temperature was measured rectally both before and after the test. (Sekunden-Thermometer Thermovit). In addition, the "old" rats were retested using a water temperature of 30°C. For this experiment ten animals each per age group 4, 27, 31 months were tested.

3) Tilting-plane test

This test predominantly measures balancing reactions. Using the method developed by Arvola *et al.* (1958) the rats were placed on a board with a rough surface (27 × 41 cm) with 60 cm high sidewalls. The animals needed to try to maintain their balance while the surface was tilted from the horizontal position – from 0° to 90° in three to four seconds time. Here the angle at which the animals could no longer prevent sliding off the board was measured. The arithmetic mean of the three tests shows the performance of an individual. In addition, the rats' ability was measured at a stable 50° angle. For this test 15 rats each from the age groups 4, 12, 16, 24, 27, 31 months were tested.

4) Horizontal-wire test

This test experimentally shows the occurrence of a decrease in muscular strength performance ability with progressive age. Here the rats were hung by their front paws on a rod which was 0.4 cm in diameter and 83 cm long

and which was fastened 43 cm over a table top. The duration time of hanging was measured for each animal using two or four extremities or also for aiding itself through using its tail. The tests lasted 60 seconds. Out of the three collected values measured per animal, the longest duration was the value used for evaluation. A resting pause of approximately five minutes between consecutive attempts was allowed. For this test 16 animals each per age group 4, 12, 20, 22, 24, 27, 30, 31 months were used.

5) Chimney test

Analogous to the method developed by Boissier *et al.* (1960) the rat had to climb backwards out of an upright pipe. The pipe measured four cm in diameter, sixty cm on length and was made of plexiglass. The inside of the pipe was grooved to allow better traction. With the pipe placed in a horizontal position, the rats first had to walk to the end opposite the opening. The rat takes up one third of the pipe. The pipe was then gently turned to 90° angle so that the animals had to climb 40 cm up the pipe backwards. The amount of time it took until the rat pulled its head out of the opening was measured. The arithmetic mean was derived from three test values. For this test, 15 animals each per age group 12, 16, 20, 24, 27, 31 months were used.

6) Climbing test

In this test the rats climbed up a vertical ladder 60 cm long and seven cm wide with the rungs spaced four cm apart. The motivation for climbing consisted of reaching a dark goal box at the top of the ladder and in avoiding falling into a cage (lined with bedding) which was located 90 cm under the ladder. Before the test, the rats were given two to three trial runs in which to learn to do the task. Data for this test consisted of registering the number of grasps with the fore paws and the amount of time required to climb the ladder after deducting the time spent resting. From these measurements one can calculate the stepping speed and the stepping width. For each animal three test runs were evaluated, in which a one to two minute rest was allowed between two consecutive tests. The mean of these three values was then calculated. For this test, 20 animals of each age group 4, 10, 14, 16, 20, 24, 27, 32 months were used.

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7) Rotarod test

Rats, placed on a rotating cylinder, needed to walk in the direction opposite that of rotation. The cylinder (5 cm in diameter, 20 cm long) was fasted 34 cm over a table top and was of a light construction. A motor allowed for the continuous setting of the rotation speed (10 rpm, 20 rpm, 40 rpm). Test arrangement: On the first day the rats spent two minutes on the slowly rotating cylinder (10 rpm). On the second and third days, the rotation speed of the previous day was doubled (20 rpm, 40 rpm). The criterium for the performance for the sample of rats used was that 60% stayed on the cylinder for 120 seconds. The parameters were each animal's frequency of starts and the amount of time spent on the rod before falling off. The animals were given five minute rests between starts. The number of starts was limited to 15 per day.

The H-Test developed by Kruskal-Wallis was used for the statistical ensurance of the results of test 1, *Spontaneous Activity,* in which several independent groups of research animals were compared. In comparing two independent age groups in Tests 2 through 7, the U-Test developed by Wilcoxon, Mann and Whithney was used. Results were regarded to be statistically significant if $p \leq 0.01$.

RESULTS

Figure 1 shows the survival curve, with the 50% survival rate at 27 months. The Gompertz curve is linear and indicates that the rats tested were aging individuals (Figure 2). For the animals that died after the age of 27 months, a slow weight loss was statistically observed approximately 100 days before death. In animals with a short life span (age at death less than 27 months) this weight loss appeared 50 days before death and increased sharply 14 days before death. It can be assumed that pathological processes influence the body weight and shorten the life span of the animals that died before the 50% survival rate of age 27 months, Figure 3 (Everitt, 1957; Coleman *et al.*, 1977; Deerberg *et al.*, 1980; Yu *et al.*, 1982).

1) Spontaneous activity

Figure 4 shows that the average spontaneous activity does not differ greatly for rats of different ages. The values show the activity data averaged over three days and aggregated over each 12-hour dark phase. It can be seen that all means lay within the confidence in-

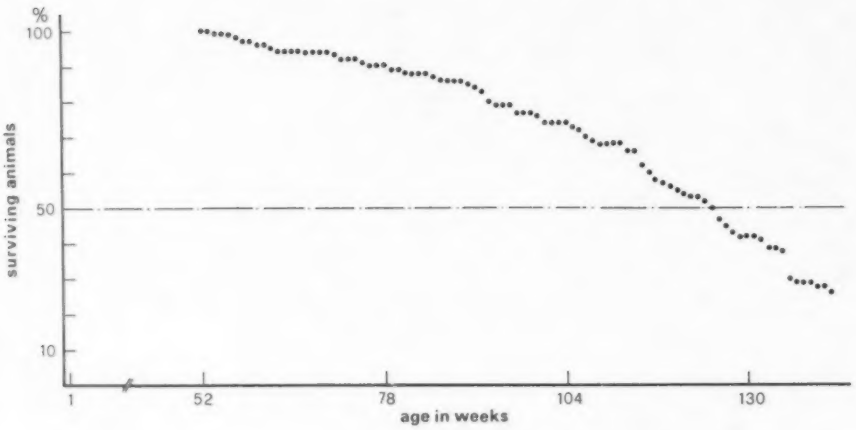

FIG. 1. Survival curve of 250 female Wistar rats. The survival rate is 125 weeks (27 months).

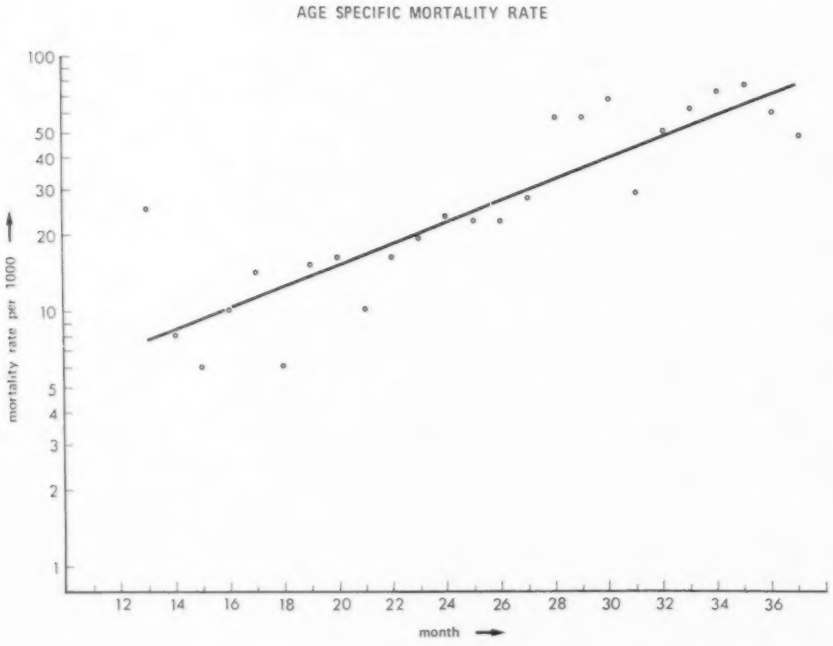

FIG. 2. Gompertz curve of 250 female Wistar rats (breeder: Hagemann, Bösingfeldt).

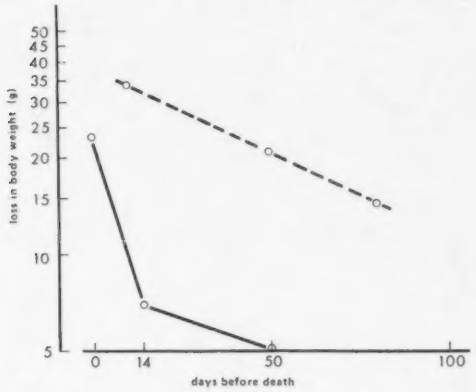

FIG. 3. Decrease in body weight beginning at 100th and 50th day before death related to the maximum body weight. —: rats which die before 27 month of age; ···: rats which die after 27 month of age.

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tervals. The regression of motility on old age shows a decreasing trend but is statistically not different from zero ($p > 0.01$). A Kruskal-Wallis Test for differences between several independent samples did not reach a level of significance at the one percent level. The activity distribution over a 24-hour period also showed a similar time course for both young and old animals with two maxima at 9 p.m. and 6 a.m.

2) Swimming test

As can be seen in Figure 5, the swimming performances of young and old rats did not differ in vigor or success during the entire testing period (U-Test, $p > 0.01$). For reasons

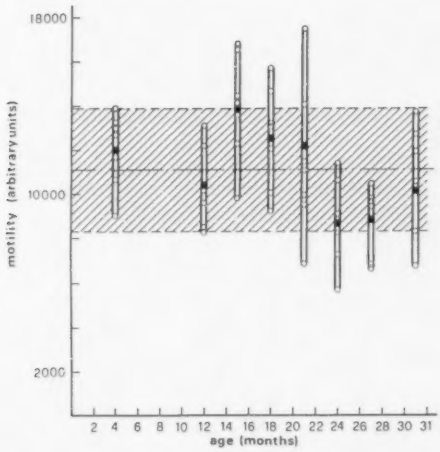

FIG. 4. Spontaneous motor activity in rats of different ages during 12 hour dark phase averaged over three days. Measurement during darkness phase (7 p.m. to 7 a.m.). White circles = individual animals, black circles = average of each age group. Shaded area: mean +/− S.D. for all age groups; n = 7 each.

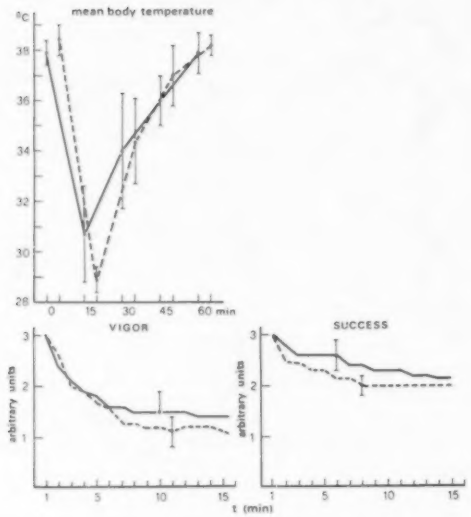

FIG. 5. Swimming performance (vigor, success) and body temperature in rats of different ages. 4 months old = ··· (n = 10), 27 months old = —(n = 10) Mean +/− S.D.; S.D. for vigor and success marked in one example for each group.

of clarity the standard deviations per minute are displayed only once each. Both young and old rats registered a strong loss in body temperature. Both groups, however, were back to their original temperatures 45 minutes after the tests were over. The additional swimming test using water which was 30°C yielded no new results other than that body temperatures did not drop as much.

3) Tilting-plane test

As can be seen in Figure 6, the ability to react quickly to an abrupt change in the surroundings by using compensating balancing movements clearly declines between the age of six and 12 months and again between 24 and 26 months. The difference between 24

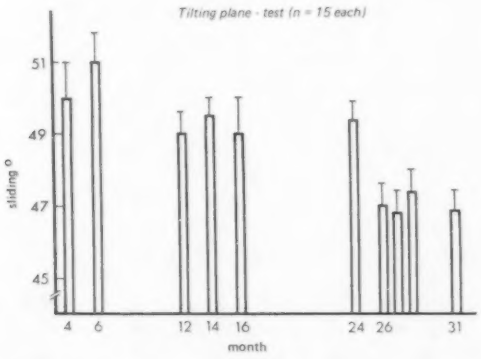

FIG. 6. Performance of rats of different ages in the tilting-plane test. Mean +/− SEM; n = 15 each.

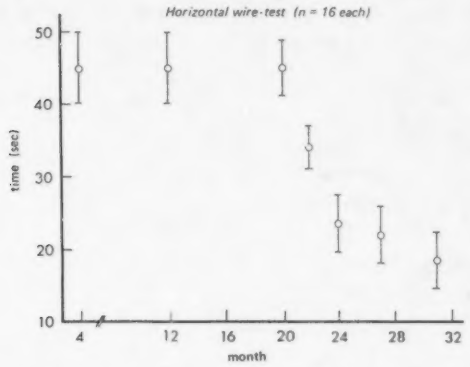

FIG. 7. Performance of rats of different ages in the horizontal wire test. Mean +/− SEM; n = 16 each.

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and 26 month old rats is significant (U-Test, $p < 0.01$). An additional test of the balancing ability using a stationary 50° tilted plane showed that older rats could perform just as well as younger rats. Consequently, the poorer performance of older rats on the first test must deal more with the ability to handle a quickly changing surrounding and in being able to react quickly enough.

4) Horizontal wire test

The results of this test (Figure 7) demonstrate that the ability to remain on a rod (maximum 60 seconds) strongly declines in rats after the age of 20 months. The difference between 20 and 24 month old rats is statistically significant (U-Test, $p < 0.01$). The decline in performance here cannot only be due to a decrease in muscular strength; the unsuccessful attempts by the older rats who used all four paws and/or their tails also point to the presence of a loss of coordination in the extremities.

5) Chimney test

The results of the Chimney test are similar to those of the Climbing test (Figure 8) in that old rats required twice as much time as the younger ones. The differences between 4 and 12 and between 20 and 27 month old rats are significant (U-Test, $p < 0.01$). This ef-

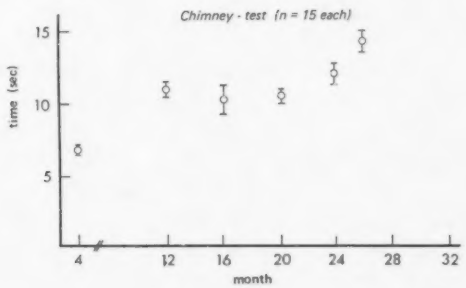

FIG. 8. Performance of rats of different ages in the chimney test. Mean +/− SEM; n = 15 each.

fect could possibly be due to the already mentioned irregularities in the coordination of the extremities. The old rats also showed signs of exhaustion sooner than their younger counterparts.

6) Climbing test

Figure 9 clearly demonstrates that the climbing time of old rats was twice as long as that of four month old rats. This decline in performance ability is due to a combination of both decreasing stepping distances and a reduction in stepping speed. In addition, the old animals showed more instances of mistakes, repeating grasps or placing the front paws on top of each other. It was also observed that the old rats displayed a discontinuous course of movements of the front and hind paws. They pulled their bodies up with their front paws and then brought up their hind legs afterward. Intra-individual variability in all age groups during the three tests was notably small. The difference in the climbing time between 4, 20 and 27 month old rats is statistically significant (U-Test, $p = 0.01$).

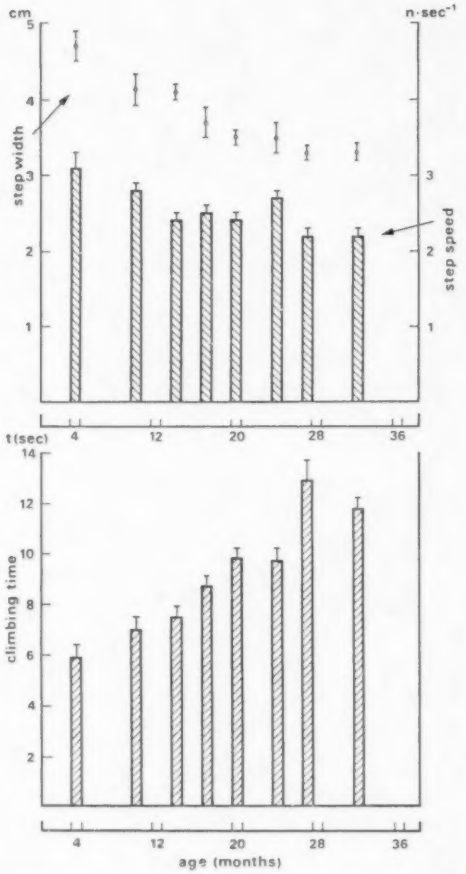

FIG. 9. Climbing performance in rats of different ages. Top chart: the step width and step speed of the forelegs; bottom chart: climbing time. Mean +/− S.D.; n = 20 each.

7) Rotarod test

Figure 10 shows that 24 month old rats already need more starts in order to complete the test than the younger rats (at 10 rpm; U-Test, $p < 0.01$). The high number of starts at the beginning of the test is due to the reaction to an unfamiliar test situation. Here the old rats especially showed great agitation. The 27 month old rats required three times as many starts as their four month old counterparts. This effect is still present at the second rotation speed (20 rpm, Figure 11). For the third rotation speed (40 rpm, Fig. 12) 20 month old rats already show less proficiency (U-Test, $p < 0.01$). For rats of all ages one could observe increased learning from day one to day three. Older rats, however, seemed to learn more slowly. Consequently, the old rats, with the exception of the oldest group, are capable of accomplishing this task. However they need more training than the younger animals for the more difficult conditions. A re-examination of the rats' behavior on this test 8, 13 and 18 days after the initial three-day course of tests revealed that for all age groups, their abilities on this complex task are not stabile without practice (Figure 13).

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DISCUSSION

The results reveal that with the help of the test batteries used, an age-dependent, graduated and differentiated change in motor skills in rats can be demonstrated in a cross-sectional analysis.

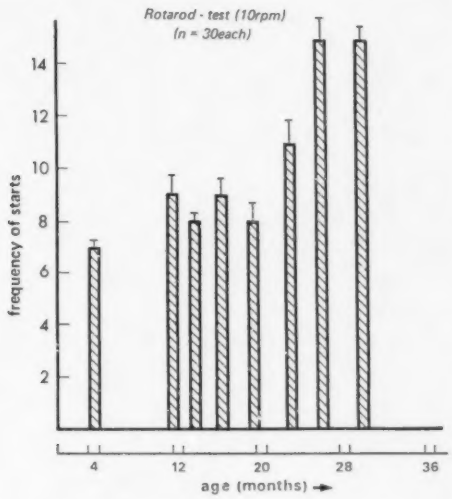

FIG. 10. Rotarod performance of rats of different age—frequency of starts for a rotation speed of 10 rpm until the desired criterium was met on the first day of training (4, 24, 27 months). Median values +/− SEM; n = 30 each.

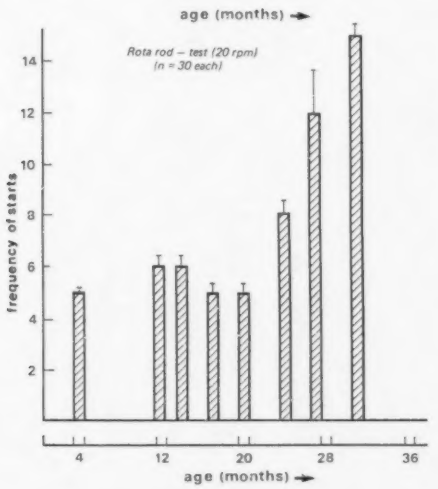

FIG. 11. Rotarod performance of rats of different age—frequency of starts for a rotation speed of 20 rpm until the desired criterium was met on the second day of training (4, 24, 27 months). Median values +/− SEM; n = 30 each.

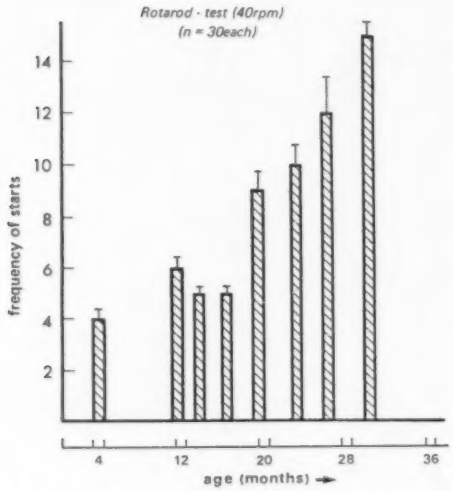

FIG. 12. Rotarod performance of rats of different age—frequency of starts for a rotation speed of 40 rpm until the desired criterium was met on the third day of training (4, 24, 27 months). Median values +/− SEM; n = 30 each.

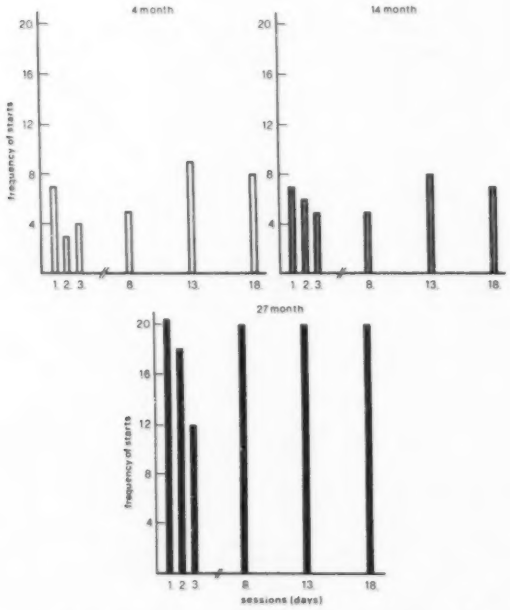

FIG. 13. Rotarod performance of rats of different age—frequency of starts on three successive days with increasing rotation speed (10, 20, 40 rpm), and at follow up tests on the 5th, 10th and 15th day after the end of training (40 rpm). Median values; n = 30 each.

1) Spontaneous activity

Spontaneous activity is attributed great significance as an indicator of the general condition of a laboratory animal in numerous neurophysiological and pharmacological experiments. The findings regarding age-related changes in spontaneous activity are divergent. Farner *et al.* (1961) reported a clear difference between 4 and 32 month old Wistar rats while Koning-Verest *et al.* (1980) showed a difference between 3 and 12 month old rats. The discrepancies with the results presented here are probably due to the measuring techniques and experimental design. For example, only primary exploratory activities are registered if measurements are only recorded for a short period of time and if the animals have not had time to adapt to the test situation (Farner *et al.* 1961; Koning-Verest *et al.*, 1980). The Koning-Verest's *et al.* (1980) results show that the measurements of individual activity can be considerably different from those of groups of rats. No significant difference was observed between 12 month male Wistar rats and older rats. Similar results were obtained by Hofecker *et al.* (1974; 1978). independent of whether several animals were researched at the same time or singly. In the latter case the activity of two individuals was simultaneously registered from a recording apparatus; the animals, however, had no visual contact with each other (Hofecker *et al.*, 1974; 1978). In interpreting the difference between the results of the above-mentioned study and this one, one needs to keep in mind these age-related deviations are only significant at a five percent level.

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2) Swimming test

The swimming test is predominantly used in animal research for studying stress resistance, endurance, and the effects of training (Bättig *et al.*, 1961; Molinengo *et al.*, 1976). Kay *et al.* (1958) observed differences in performance between the sexes and between old (23–27 months) and young (7–8 month) rats (using Sprague Dawley rats). Norimura *et al.* (1978) found an age-dependent decline in ability in male mice. Marshall *et al.* (1979) observed the same age-related differences using male Fischer 344 rats with the same evaluation scale that was used in this study. In comparison to the results of that study, however, the rats in the present study registered considerably higher values in the evaluation scale. However, qualitatively the young rats differed from the old rats in that they moved quite intensely during the first five minutes and thus were more quickly wet. The old rats were in general much calmer and spent much of the time floating; this behavior was even more pronounced using the higher water temperature (30°C). It is possible that the old animals had advantages in that they had more body fat and a greater fur mass. The behavior of the old rats could be interpreted differently. The old rats could have developed a different strategy than the younger animals and thus have spent the same duration of swimming time in a qualitatively different swimming behavior. It is possible that the differences between the different age groups would be preserved under more difficult conditions, for example, if they were required to achieve a goal as quickly as possible or under stress. However, as far as simple swimming movements are concerned, that is, being able to successfully cope in water for a certain amount of time, no decline in performance was observed.

3) Tilting plane test

The ability to successfully react to a changed physical environment by adapting the body position to it is largely preserved in rats up to the second year. Uncertainty appears

only in older rats. Since it is known that the speed of nerve impulses and the vestibular reactions are hardly impaired in old age (Birren *et al.*, 1979; Wallace *et al.*, 1982) and since Wallace *et al.* (1980) also found little deterioration of the movement reflexes in old rats in a series of tests, the decline in quick balancing movements found in old rats on this test could be attributed to a deterioration of vigilance regulation.

4) Horizontal wire test

In this test 20 month old rats begin to show signs of what turns out to be a progressive decline in performance ability. This process is similar to the loss of muscular strength in aging humans (Fischer *et al.*, 1947). However, loss of coordination certainly also plays a role here (Shock *et al.*, 1970). In testing senile mice on the horizontal wire test Miquel *et al.* (1978) found a loss in muscular strength as well as a weakening of the neuromuscular coordination. The unsuccessful attempts of the rats to stay on the wire by using all four extremities and their tails indicate this sort of deterioration in their mobility.

5) Chimney test and 6) Climbing test

In both of these tests the course of decline in motor abilities is similar although the movement patterns are not qualitatively the same. This decline can be noticed at 12–13 months and becomes progressively more apparent with increasing age. It is interesting to note that the old organisms make mistakes in spite of reduced speed. These mistakes could possibly be due to rigid goal fixation as Brinley *et al.* (1965) and Welford (1959) showed in their analysis of the relationship between precision and speed of movements in humans. This hesitant behavior in old rats can be seen quite well in their shorter stepping distances and in that they rest more often. If muscular strength were also a requirement here, the declining abilities would mainly be caused by a lack of coordination. This is in agreement with the results of the horizontal wire test in that a measurable decrease in muscular strength is first noticable in 22 month old rats. The unequal weight distribution of the individuals and the small within-group variance speak against differences in body weight having caused these differences.

In summary there is a hierarchical organization of performance changes across individual tests. The schematic presentation is obtained by taking a percentage of the change in performance using the values of the young animals (four months) as a reference (Figure 14). The criterion here is simply the time point at which the changes due to aging appear. Spontaneous activity (the expression of fundamental life processes such as eating, drinking, cleaning oneself, moving around, etc.) and swimming are equally unaffected by aging. Current knowledge about control mechanisms and sensomotoric control of behavior suggest that the movements involved in spontaneous activity and swimming are controlled by phylogenetically old spinal and cerebral structures. These are possibly less susceptible to deterioration in old age (Brunner *et al.*, 1973; Wallace *et al.*, 1980; Berntson *et al.*, 1976). One year old rats only show a slight impairment of their ability to maintain their balance. This impairment, which probably reflects an increase in reaction time, advances only in insignificant degrees with increasing age.

These results, the slight changes in the speed of nerve impulses, an increase in reaction time and an only slight deterioration of the reflexes (Wallace *et al.*, 1980), are thus in agreement with each other. In contrast, bodily strength, as shown here in the horizontal wire test, is maintained for a long period of time. However, after 20 months of age there is

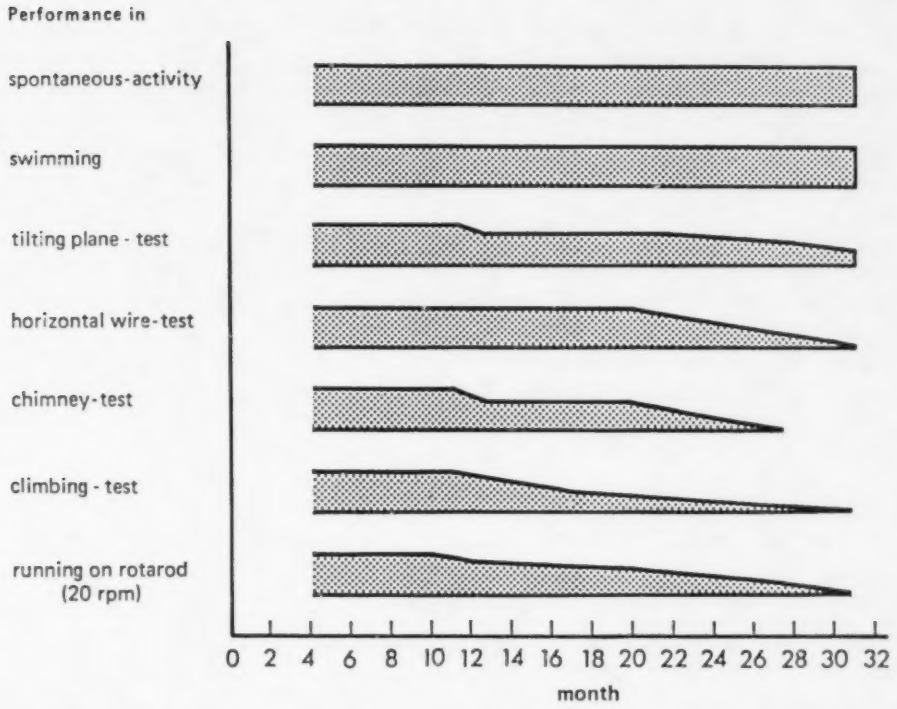

FIG. 14. Summary of changes in motor performance with increasing age in reference to performance of 4 month rats.

a drastic decline in bodily strength. Many experiments point to strong changes in various muscles or groups of muscles in old age. The declines in performance which occur very early and which are very pronounced, are obvious in the more complex tasks, such as in the climbing, the chimney and the horizontal wire tests. Here the causes can be found in a decrease in the functions of the central nervous system and cerebral area of movement control as well as in the peripheral region of the anatomical areas involved in movement.

Reasons for this include deterioration of the neuro-muscular transmission of information (Reis *et al.*, 1977; Peng *et al.*, 1979; Maggi *et al.*, 1979), reduced metabolism in the muscle cells, diminished supply of energy reserves (Ermini, 1974), and degeneration of the muscle fibers (Marhawa *et al.*, 1980). The findings and evaluations of the peripheral structural changes in old age are, however, not uniform. This especially applies to the old rats that are quite capable of adapting, performing and of maintaining endurance. In comparison to their younger counterparts, however, the old rats require more practice and time in order to be able to perform the task.

In any case, this decline in motor abilities in old rats did not show strong discontinuities, but rather occurred in asynchronous and graduated steps. It is of interest to compare this development with Ribot's (1892) "first in last out" theory, which Wallace *et al.* (1980) proposed in their study. According to this theory, the motor abilities which are ontogenetically late-developed are more susceptible to deterioration than those developed

early. An ontogenetic development of locomotion in young rats has been shown by Altman *et al.* (1975). The results obtained in the present experiment show a reversal of this process in old age, and thus are consistent with the theory. Young rats are able to walk quadrapedically at ten days of age. This depends on the development of the ability to move the hind extremities. It is, however, not until the 18th to 20th day that they are able to climb or walk on a rotating rod. The fact that the complex pattern of movement is only later available is obviously related to the relatively late maturation of the cerebellum. In an experiment using similar test batteries, Brunner *et al.* (1973) found a relationship between an experimentally damaged cerebellum and the occurrence of a loss of coordination in an animal. They also came to the conclusion that there is a hierarchical organization of the susceptibility of motor abilities.

One needs to consider the consequences the differentiation of functional changes in old age has on the vitality of an old animal. This vitality indicates a complex variable pertaining to the condition of the functional capacity of an organism to maintain its vital needs and to show a stable reaction to endogenous and exogenous stimuli. The results of this experiment brought us to the conclusion that an elderly rat, in spite of declining motor abilities, is flexible enough to handle the demands placed on its motor abilities. Thus, the loss of vitality in old age is much less dramatic than postulated by Beier *et al.* (1973).

SUMMARY

In the present paper the degree of motoric change was studied in female Wistar rats of various ages. In characterizing the population a survival curve (over a three year period) and a Gompertz curve were established. In addition, the development of the body weight was determined. From these it was determined that the population studied was old, the 50% survival rate being at 27 months, and that the development of the body weight before death followed a different course for animals with short as opposed to animals with long life spans. In order to be able to study the motor abilities of rats of various ages a test battery was used which demonstrated varying complexity in patterns of behavior: spontaneous activity, swimming test, climbing a vertical ladder, horizontal wire test, tilting plane test, chimney test and rotarod test. The results show that in comparison to younger rats, older rats showed differentiated decline in performing progressively more complex tasks. There were no recognizable age-related differences in the measurement of spontaneous activity and in the swimming test. In the climbing and in the chimney tests clear deviations were apparent from 20 months and older; in comparison, the movement abilities of older rats showed a much less dramatic decline in the horizontal wire test. Decline in reaction time first became apparent in two year old rats in the tilting plane test. In the rotarod test the lack of coordination became especially prominent after 24 months of age. These findings were set in relation to central and peripheral structures and functions involved in motor control. Further, the results were discussed in the context of a continuous performance profile in its relation to chronological age.

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AGE-RELATED CHANGES IN CATALASE AND PEROXIDASE ACTIVITIES IN THE EXCISED LEAVES OF *ELEUSINE CORACANA* GAERTN. CV PR 202 DURING SENESCENCE

K.B. KUMAR and P.A. KHAN

Department of Botany, Berhampur University Berhampur-760 007, Orissa, India

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Abstract—Changes in the activities of the enzymes-catalase and peroxidase were studied in the excised leaves of ragi (*Eleusine coracana* Gaertn. cv PR 202) plants belonging to different ages. Catalase exhibited a positive and peroxidase a negative correlation with the changes in chlorophyll. Catalase and peroxidase were negatively correlated with each other. Peroxidase exhibited an age-related drift in its activity. Kinetin could maintain the levels of chlorophyll and catalase, and also caused an increase in peroxidase activity. Both indoleacetic acid and gibberellic acid had no effect on the changes of chlorophyll but increased peroxidase activity. Catalase levels were maintained by indoleacetic acid but gibberellic acid had no effect on this enzyme.

INTRODUCTION

HYDROGEN PEROXIDE is synthesized in the plant and animal tissue by the transfer of electrons to molecular oxygen by different oxidative enzymes (Brennan *et al.*, 1979). In spite of decomposition and utilisation by the enzymes catalase (EC. 1.11.1.6) and peroxidase (EC. 1.11.1.7), a steady state of H_2O_2 is usually maintained (Boveris *et al.*, 1972; Chin and Frenkel, 1976; Grodzinski and Butt, 1976; Sagisaka, 1976). Such a maintenance in the levels of H_2O_2 indicates that catalase and peroxidase are not involved in complete destruction or utilisation of H_2O_2 formed; rather a balance is maintained between the synthetic and decomposing enzymes involved in H_2O_2 metabolism. The onset of senescence is believed to cause an imbalance in the levels of H_2O_2 and an increase in endogenous H_2O_2 is considered as one of the factors in fruit (Brennan and Frenkel, 1977) and leaf (Parida *et al.*, 1978) senescence.

Varying responses of the enzymes catalase and peroxidase have been reported from different plants during senescence. Hence the turnover of H_2O_2 in regulating the process of senescence has been stressed (Brennan and Frenkel, 1977; Parida *et al.*, 1978; Birecka *et al.*, 1979). Patra *et al.* (1978) considered catalase as a reliable indicator of senescence. The biochemical function of peroxidase is not clear as the *in vivo* substrate and the source of H_2O_2 are not yet identified (Fric, 1976; Braber, 1980). The relationship between these two enzymes involved in the utilisation and decomposition of hydrogen peroxide is not clear. Therefore, the present investigation has been designed to study the role of catalase and peroxidase in the leaves of ragi *(Eleusine coracana)* plants of different ages. Further, the

involvement of the enzymes in H_2O_2 turnover, their response to the growth regulators and their relationship have been studied.

MATERIALS AND METHODS

Seeds of ragi (*Eleusine coracana* Gaertn. cv PR 202) were grown in experimental pots and the first, second, third and fourth leaf of 7, 10, 15, and 20 days old plants respectively were used for experimentation. Excised leaves were thoroughly washed and leaf samples belonging to different age groups weighing ca. 200 mg were floated in separate petri dishes containing distilled water. Third leaf from 15-day-old plants were detached, washed and floated in separate petri dishes containing 50 ml solutions of kinetin (5 mg/l), indoleacetic acid (20 mg/l) and gibberellic acid (100 mg/l). All the petri dishes were incubated in the dark at room temperature 28 ± 2°C. All manipulations were done in asceptic conditions. Samples were taken initially and at intervals for chlorophyll and enzyme analysis.

Chlorophyll was extracted thrice with 80% (v/v) boiling ethanol. Chlorophyll content of the extracts was determined by measuring the absorbance of the combined ethanolic extracts at 665 nm.

For enzyme extraction leaf material was ground with 0.1M phosphate buffer pH 7.0 in a prechilled mortar and pestle and the homogenate was centrifuged at 15,000 × g at 4°C for 30 minutes and the aliquot was used as the source of the enzymes.

Catalase activity was assayed by a modified method of Braber (1980). One ml enzyme extract was added to 2 milliliters 0.005M H_2O_2 and three ml phosphate buffer pH 7.0. The reaction was stopped by adding 10 ml 0.7N sulphuric acid after one minute incubation at 20°C. The residual H_2O_2 was titrated with 0.01N $KMnO_4$. A blank was prepared by adding the extract to an acidified solution of the reaction mixture at zero time. Catalase activity was expressed as mmol H_2O_2 used per gram fresh weight of leaf.

Peroxidase activity was assayed following the modified method of Kar amd Mishra (1976). Assay mixture for peroxidase contained 2 ml 0.1M phosphate buffer pH 7.0, 1 ml 0.005M H_2O_2 and one ml well diluted enzyme extract. The reaction was stopped by adding to the reaction mixture one ml 1.5N sulphuric acid after 5 minutes incubation at 25°C and the amount of purpurogallin formed was estimated by measuring the absorbance at 420 nm. The enzyme activity was expressed in absorbancy units.

RESULTS AND DISCUSSION

Changes in the levels of chlorophyll and the trends in the activities of the enzymes catalase and peroxidase in the excised green and mature leaves from 7, 11, 15 and 20 days old ragi plants are shown in Figure 1. Maintenance of maximum and constant levels of chlorophyll and protein (for a period of at least two days) was considered as the index of leaf maturation. First, second, third and fourth leaf from base upwards were green and mature respectively in 7, 11, 15 and 20 days old plants; other leaves at these ages were either developing or senescing. As the first leaf is from the youngest plant and in the increasing order the fourth leaf is from the oldest plant (under the experimental conditions), the leaf number that is first, second, third and fourth referred to in this paper indicate the age of plants.

There was a decline in the levels of chlorophyll in the leaves from all ages. First and second leaf took two days to senesce (reach minimum levels of chlorophyll) whereas the third and fourth leaf took three and four days to senesce respectively. There was a decline in catalase activity in all the leaves. The enzyme ran a parallel course to chlorophyll degradation. The activity of the enzyme in the first, second, third and fourth leaf took two, two, three and four days respectively to reach minimum levels. There was a coincidence in the number of days taken by the enzyme and chlorophyll to reach mimimum levels. The declining trends in catalase activity corroborated with the reports from rice leaves (Kar and Mishra, 1976; Parida *et al.*, 1978) and do not agree with the reports of rise in enzyme activity in barley and wheat leaves (Farkas *et al.*, 1964; Kisban *et al.*, 1964). Such opposite trends may be due to species specificity (Patra *et al.*, 1978).

Peroxidase exhibited opposite trends to chlorophyll and catalase. There was a rise in the activity of peroxidase in all the categories of leaves. The peak of peroxidase activity cor-

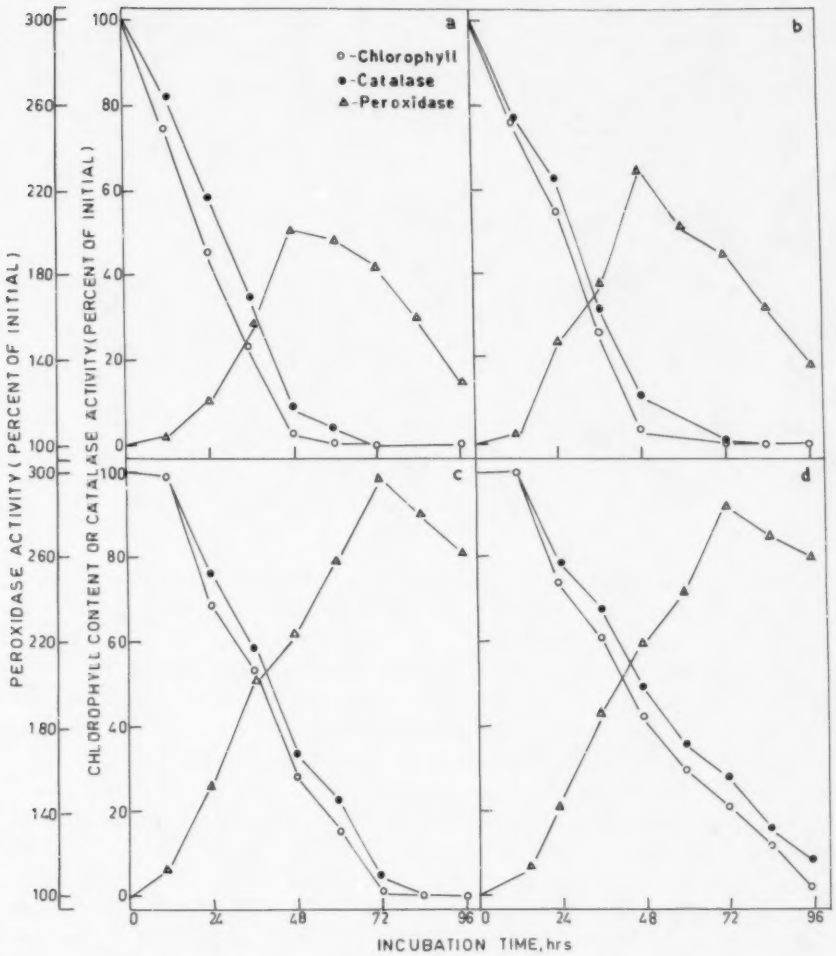

FIG. 1. Changes in the levels of chlorophyll, and activities of catalase and peroxidase in the detached 1st(a), 2nd(b), 3rd(c), and 4th(d) leaf of ragi during senescence.

responded to the minimum levels of catalase and chlorophyll in all leaves except the fourth one. There was a decline in the activity of peroxidase after reaching the peak. In the fourth leaf the decline started when the levels of catalase were still maintained (Figure 1).

The correlation between the trends in chlorophyll and catalase is shown in Figure 2a. There was observed a close positive correlation between the decrease in chlorophyll and catalase activities. Such a correlation was also reported from rice leaves (Kar and Mishra, 1976). The correlation between peroxidase and chlorophyll degradation is shown in Figure 2b. A close correlation was also observed between peroxidase and chlorophyll breakdown in the leaves of all ages but in a negative way. Birecka *et al.* (1979) did not observe any correlation between increase in peroxidase and rate of chlorophyll breakdown in oat leaves.

The activities of the enzymes catalase and peroxidase did not show similar pattern of changes in different leaves. The first leaf showed minimum levels of catalase within two

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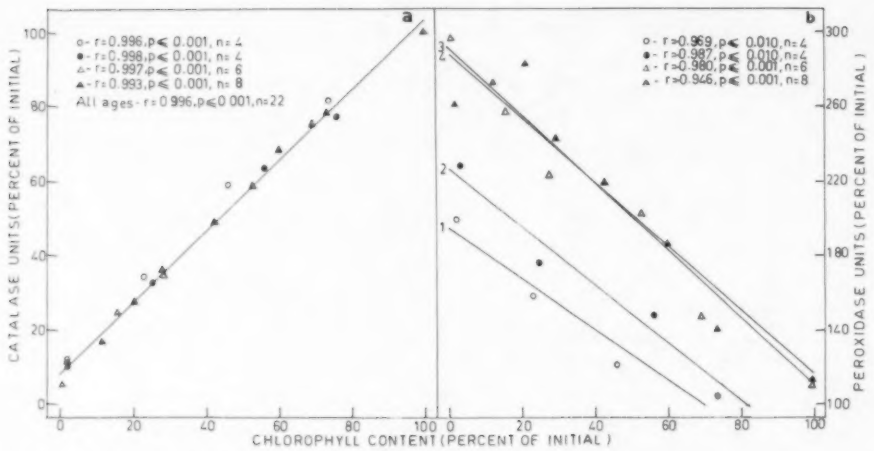

FIG. 2. Correlation of catalase (a) and peroxidase (b) activities with the chlorophyll content in the detached 1st (○), 2nd (●), 3rd (△) and 4th (▲) leaf of ragi during senescence. The numbers 1, 2, 3 and 4 indicate the lines of regression for the 1st, 2nd, 3rd and 4th leaf respectively.

days where as the fourth leaf took four days to reach minimum levels. Peroxidase also exhibited similar behaviour, the first and second leaf taking two days to reach the peak whereas the third and fourth leaf three days to do so. The correlation between the changes in catalase activity and chlorophyll content in the leaves of different ages could be expressed with a single regression line. On the other hand, though activity of peroxidase showed a close negative correlation with chlorophyll degradation in the leaves of all ages, it could be expressed only by using different regression lines, one for each age (Figure 2). This clearly indicated that with the increasing age there was no change in the relation between catalase and chlorophyll, whereas peroxidase exhibited an age dependent drift in its activity in relation to the changes in chlorophyll, but still maintained a correlation with the latter.

Earlier reports indicate that catalase from beef liver could split into monomeric units and these monomeric units develop peroxidase activity (Samejima *et al.*, 1968; Marklund, 1973). Such a conversion of tetrameric molecules to monomeric molecules was considered as the possible reason for increase in peroxidase activity in primary leaves of the bean (Braber, 1980). The present investigation indicates opposite trends in the activities of the enzymes in the leaves belonging to different ages. Regression analysis of the data pertaining to the activities of the enzymes from different leaves is shown in Figure 3. The units of catalase and peroxidase activities after the latter enzyme started declining was not included in the analysis because the decline in the peroxidase activity was believed to be due to the loss of catalase activity and at that stage where the change in catalase activity is negligible but the activity of peroxidase exhibited a gradual decline, no correlation is expected between the enzymes. In all the leaves the enzymes had a strong negative correlation. This supports the possibility of conversion of tetrameric molecules with catalase activity to monomeric molecules with peroxidase activity in the leaves from different ages.

To study the effect of different anti-senescence agents on the trends of catalase and peroxidase activities, and chlorophyll degradation, the leaves floated in the solutions of kinetin, indoleacetic acid and gibberellic acid were analysed for chlorophyll and enzyme activities. Kinetin could significantly retard the degradation of chlorophyll but in-

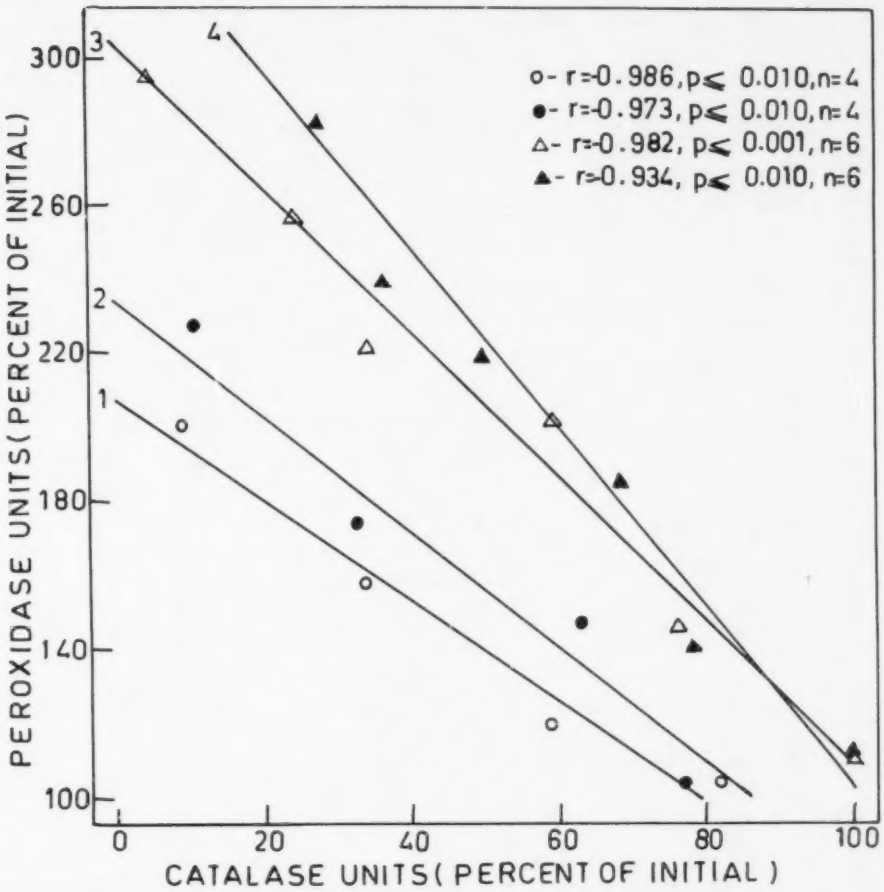

FIG. 3. Correlation between peroxidase and catalase activities in the detached 1st (○), 2nd (●), 3rd (△), and 4th (▲) leaf of ragi during senescence. The numbers 1, 2, 3 and 4 indicate the lines of regression for the 1st, 2nd, 3rd and 4th leaf respectively.

doleacetic acid could cause no effect. This was in agreement with the popular view that in herbaceous plants cytokinins are effective but auxins are not in checking chlorophyll degradation (Osborne, 1967). Gibberellin was also not effective in causing a check in chlorophyll degradation (Figure 4a).

Earlier reports indicate that kinetin and benzimidazole, the agents capable of maintaining the levels of chlorophyll, could also maintain catalase activity to a significant extent in rice leaves (Kar and Mishra, 1976). In the present investigation, kinetin, the powerful senescence retardant, maintained the levels of catalase; and gibberellic acid which was not effective in retarding chlorophyll degradation had no effect on the enzyme activity. However, the behaviour of indoleacetic acid was different. Though the growth hormone was incapable of maintaining the levels of chlorophyll, it could efficiently maintain the activity of the enzyme (Figure 4b).

If peroxidase has any functional significance in the process of senescence, kinetin is

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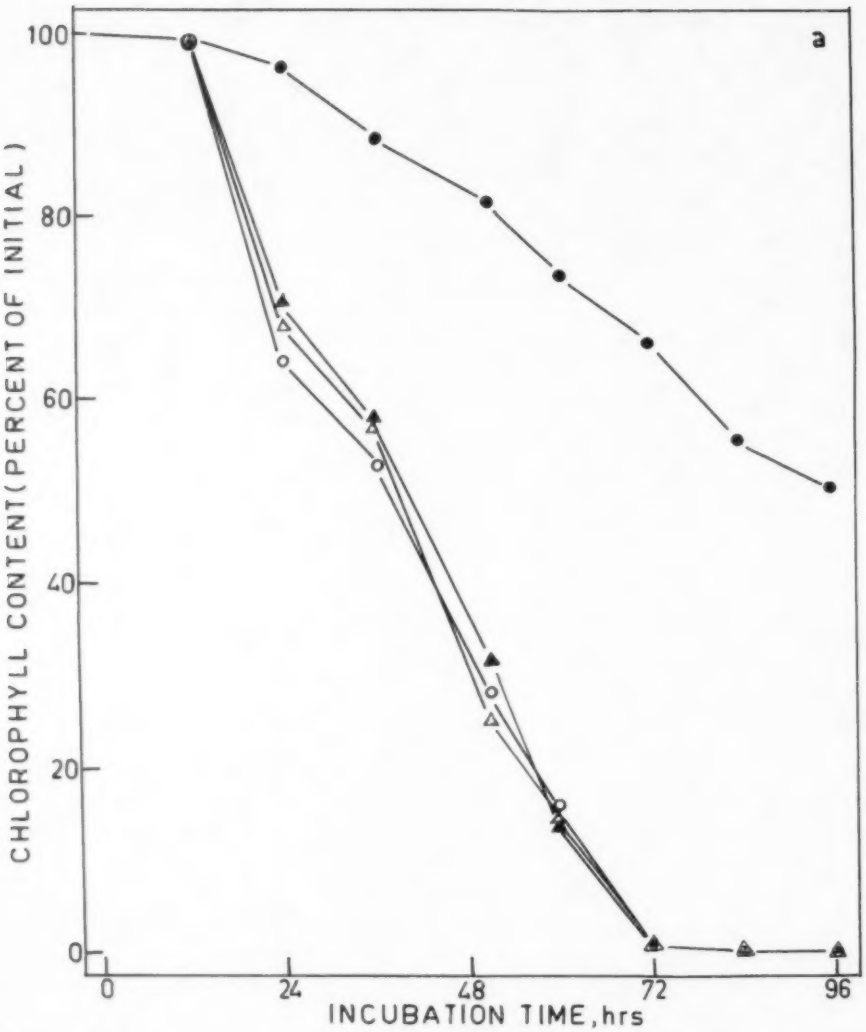
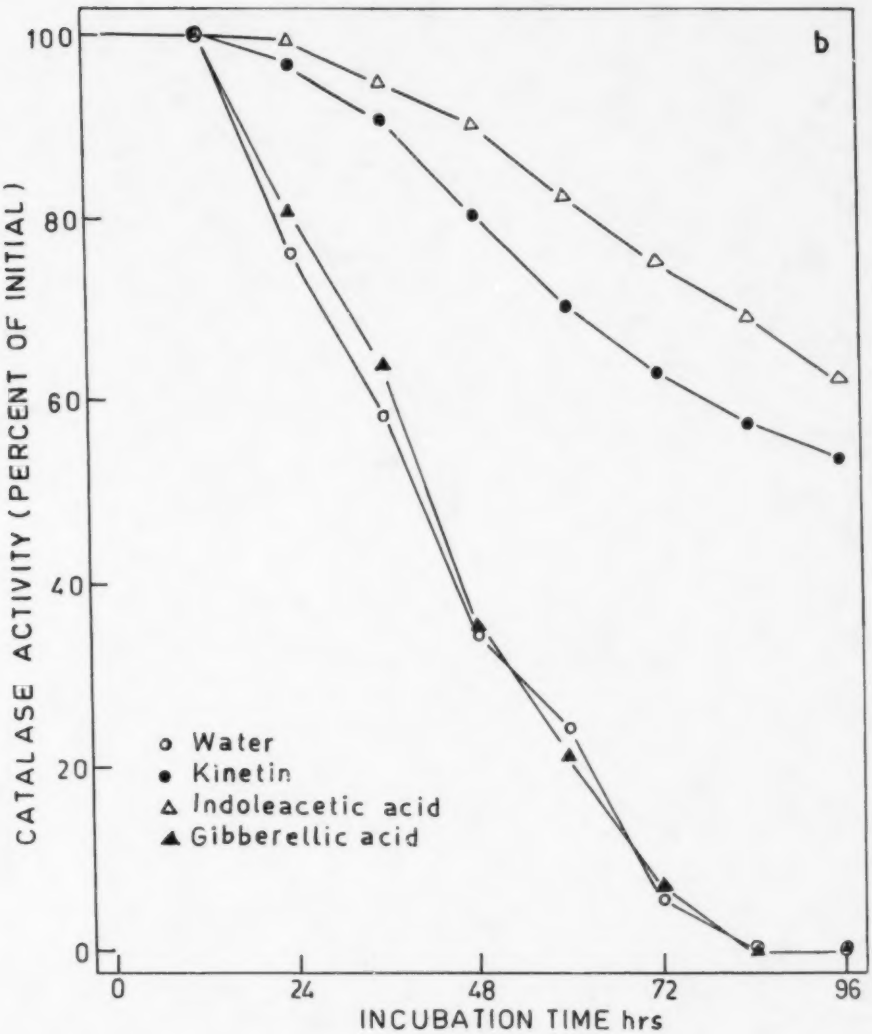


FIG. 4. Effect of growth regulators on chlorophyll content (a), catalase (b) and peroxidase (c) activities in the excised third leaf of ragi during senescence.

expected to decrease the rise in peroxidase activity induced during senescence, and indoleacetic acid and gibberellic acid are expected to have no effect. Contrary to these expectations all the three hormones caused further rise in the activity of the enzyme (Figure 4c). Cytokinin-induced rise in peroxidase activity has been observed in rice (Kar and Mishra, 1976) and oat (Birecka *et al.*, 1979) leaves. However Parish (1968) reported a cytokinin-induced fall in the enzyme activity.

There is an increase in endogenous H_2O_2 during the senescence of fruits (Brennan and Frenkel, 1977) and leaves (Parida *et al.*, 1978) and the two enzymes may control senescence by eliminating H_2O_2. Such a role of the enzyme catalase is strongly supported



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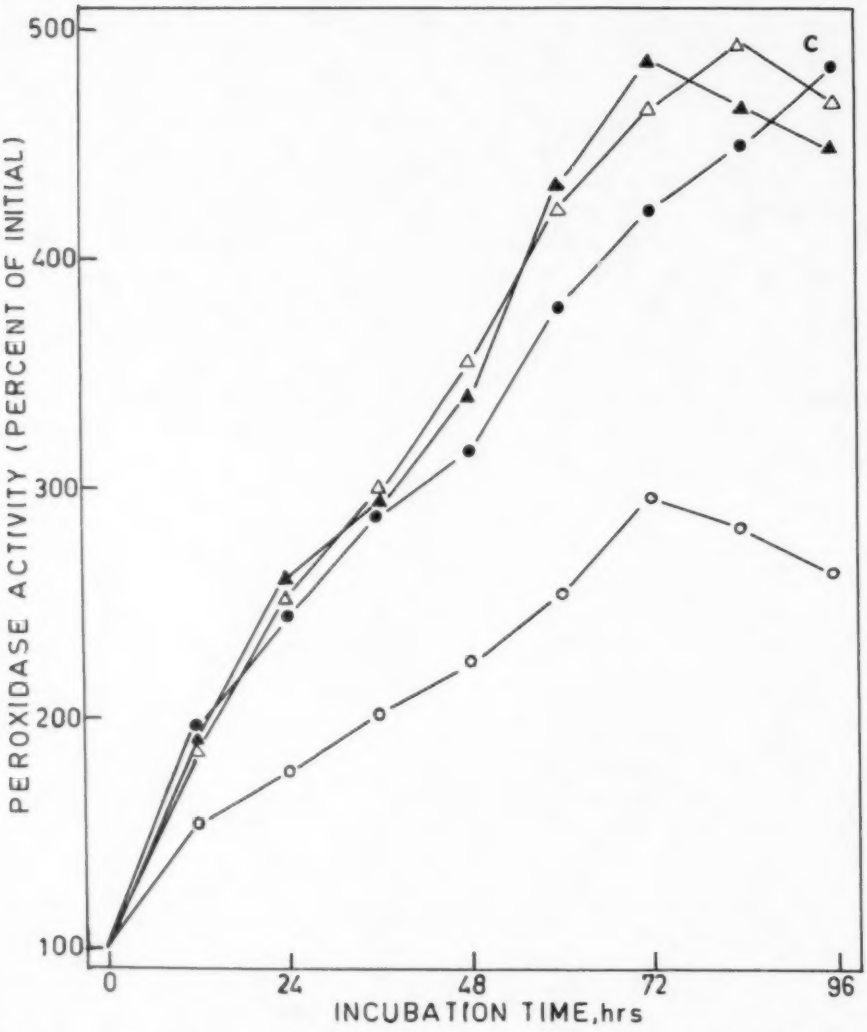
by earlier workers who found that there is a decrease in the activity of the enzyme during senescence and anti-senescence agents could significantly retard such a decrease (Kar and Mishra, 1976). The present investigation also adds support to such a view excepting the behaviour of indoleacetic acid. Decrease in catalase activity during senescence may be due to the action of proteolytic enzymes (Parida *et al.*, 1978). While Kar and Mishra (1976) considered the increase in peroxidase activity as a wound induced change, Birecka *et al.* (1979) opined that the increase is probably an induced protective reaction which could cause a delay in senescence by eliminating H_2O_2. Rudolph and Sukatsch (1968) suggested that peroxidase has a stabilizing effect on chlorophyll.

Increase in the activity of the enzyme peroxidase in the presence of growth regulators that maintain catalase activity goes against the view of the conversion of catalase



molecules to peroxidase molecules. Further, conversion of dimeric molecules lacking catalase activity to monomeric molecules with peroxidase activity is also suggested as a factor responsible for the increase in peroxidase activity (Sund *et al.*, 1967). Hence, increase in peroxidase activity does not seem to be due to a simple conversion of catalase molecules to peroxidase molecules. Some other factors (like dimeric molecules) are also involved in the process.

The present investigation however does not provide any explanation for the maintenance of catalase activity by indoleacetic acid which could not prevent chlorophyll degradation.

SUMMARY

Changes in the activities of the enzymes catalase and peroxidase were studied in the excised leaves of ragi (*Eleusine coracana* Gaertn. cv PR 202) plants belonging to different

ages. Catalase and peroxidase activities exhibited decreasing and increasing trends respectively during senescence. The former enzyme showed a positive and the latter a negative correlation with the degradation of chlorophyll. There was no change in the activity of catalase in relation to the changes in chlorophyll with the increasing age; where as peroxidase exhibited an age dependent drift in its activity. The two enzymes were inversely related and showed negative correlation. Kinetin could maintain the levels of chlorophyll and catalase, and caused an increase in peroxidase activity. Both indoleacetic acid and gibberellic acid had no effect on the changes of chlorophyll but increased peroxidase activity. Catalase levels were maintained by indoleacetic acid but gibberellic acid had no effect on the enzyme. Conversion of tetrameric catalase molecules to monomeric peroxidase molecules is suggested as a factor partially responsible for the increase in peroxidase activity during senescence.

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AGE-DIFFERENCES IN CHOLINE ACETYLTRANSFERASE ACTIVITIES AND MUSCARINIC RECEPTOR BINDING IN BRAIN REGIONS OF C57BL/6J MICE

STEVEN B. WALLER[1] and EDYTHE D. LONDON[1,2]

[1]Laboratory of Neurosciences, Gerontology Research Center,* National Institute on Aging
Baltimore City Hospitals, Baltimore, Maryland 21224

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Abstract—Muscarinic receptor binding and choline acetyltransferase, (ChAT, EC.2.3.1.6.) activity were assayed in four brain regions of C57BL/6J mice of four ages (4, 12, 18, and 24 months). In the cerebellum, there were no age differences in either of the cholinergic markers. However, significant age differences were noted in the V_{max} for ChAT and in the B_{max} for [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) in the cortex, striatum and hippocampus. Increases were noted in V_{max} of the synthetic enzyme in all three regions and for B_{max} in the hippocampus. B_{max} for [^{3}H]QNB decreased in the cortex and striatum. The high- and lower-affinity muscarinic binding constants, the percentage of muscarinic binding to high affinity sites determined by carbamylcholine displacement of [^{3}H]QNB, as well as the affinities of ChAT for acetyl coenzyme A and choline chloride showed no age differences in any brain region.

INTRODUCTION

WE HAVE previously observed that the activity of the cholinergic synthetic enzyme, choline acetyltransferase (EC.2.3.1.6., ChAT), is significantly elevated in the cerebral cortices, hippocampi and striata of 24-month old male C57BL/6J mice compared with 4-month old mice (Waller *et al.*, 1983). The objective of the present study was to determine whether these age differences in ChAT reflected changes in K_m for substrates or V_{max} for the enzyme, or whether they reflected differences in other aspects of cholinergic function. Therefore, ChAT activity as well as parameters related to muscarinic receptor binding were assessed in brain regions from mice of four ages. Because muscarinic agonist drugs bind with different affinities to several populations of receptors (Birdsall *et al.*, 1978), we investigated possible age differences in the affinities of carbamylcholine, a muscarinic agonist, for high- and lower-affinity muscarinic binding sites and in the relative proportions of these sites.

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[2]To whom all correspondence should be addressed at: Addiction Research Center, National Institute on Drug Abuse c/o Gerontology Research Center, Baltimore City Hospitals, Baltimore, Maryland 21224

*The Gerontology Research Center is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

METHODS

Male, C57BL/6J mice (Gerontology Research Center; Baltimore, MD) aged 4-, 12-, 18-, or 24 months were used in this study. The maximum life span of C57BL/6J mice is from 30 to 35 months, with a median of 22 to 27 months (Russel, 1966; Maker *et al.*, 1973). Animals were housed, six to eight per cage, in an environment of controlled temperature (25–27°C) and alternating 12 hour light (0500–1700 hours) and dark cycles. Food (24% protein, NIH formula) and water were available at all times.

Animals were decapitated, and their brains rapidly removed and dissected into regions while on an aluminum plate cooled by ice. Brain region samples were stored at −70°C until assayed.

For binding experiments, tissue samples were sonicated (Sonicator Cell Disruptor, Model W-220F, Heat Systems Ultrasonics, Plainview, NY) in 800 volumes (w/v) of 20 mM Tris-HCl buffer, pH 7.4. Assays of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding were performed using a modification of the method of Yamamura and Snyder (1974) as described by Ikeda *et al.* (1980). Tissue sonicates were incubated with L-[^{3}H]QNB (33.1 Ci/mmol, New England Nuclear Corp., Boston, MA), 10^{-12} to 10^{-8} M, for 90 minutes at room temperature. The final incubation volume was 1 ml. Incubations were terminated by filtration. Nonspecific binding, obtained in the presence of 100 μM oxotremorine, was subtracted from total binding at the various concentrations of ligand. The variation in total specific binding among triplicate samples was always less than 10% of the mean. The percent of [^{3}H]QNB bound was always less than 5% of the free ligand concentration. Estimates of total muscarinic receptor density (B_{max}) and affinity of [^{3}H]QNB for binding sites (K_{QNB}) were obtained by Eadie-Hofstee analysis of saturation isotherm data. Under the conditions of this experiment, saturation was achieved at 0.2–0.3 nM [^{3}H]QNB for the four brain regions assayed.

Estimates of the percentage of total specific [^{3}H]QNB binding that was associated with muscarinic agonist high affinity binding sites (B_{Hi}), and the affinities of agonist for high- and lower-affinity sites ($K_{Hi\text{-}Carb}$ and $K_{Lo\text{-}Carb}$, respectively) were obtained from assays of [^{3}H]QNB binding in the presence of 10^{-2}–10^{-10} M carbamylcholine chloride (Sigma), in aliquots of the same tissue sonicates used to obtain binding saturation isotherm data for [^{3}H]QNB. The concentration of [^{3}H]QNB used in these assays was 0.3 nM.

Theoretical binding curves of the competitive displacement of [^{3}H]QNB by the agonist cambamylcholine were analysed using the least squares routines of the Hewlett-Packard non-linear regression analysis program and a HP9845A desktop computer (Hewlett-Packard, Fort Collins, CO). Data were fitted to a simple mass action expression for the case of two unique binding sites for a single ligand (carbamylcholine), as described by Ikeda *et al.* (1980).

For assays of ChAT activity, tissue samples were sonicated in 20 volumes (w/v) of 50 mM Tris-HCl buffer, pH 7.4, containing 0.2% (v/v) Triton X-100. Enzyme activity was determined according to the method of Bull and Oderfeld-Nowak (1971). The effect of acetyl coenzyme A ([1-^{14}C]acetyl CoA, 2–5 mCi/mmole: Amersham Corp., Arlington Heights, Il) concentrations on enzyme activity was determined by varying the concentration of acetyl CoA (1 to 100 μM) in the assay medium, while the concentration of choline chloride was maintained at 60 mM. The effect of choline chloride concentration on enzyme activity was determined by varying the concentration of choline chloride, (0.05 to 60 mM) while maintaining the acetyl CoA concentration at 100 μM. Values of K_m and V_{max} were obtained by the Eadie-Hofstee method.

Protein concentrations in tissue sonicates used for binding assays and enzyme assays were determined by the method of Lowry *et al.* (1951). Values of B_{max} and V_{max} were reported in terms of protein.

Significance of age differences among mean values for protein concentrations of tissue sonicates, binding parameters (K_{QNB}, B_{max}, $K_{Hi\text{-}Carb}$, $K_{Lo\text{-}Carb}$, B_{Hi}) and enzyme parameters (K_m for choline chloride, K_m for acetyle CoA, V_{max}) were assessed by one-way analyses of variance and Bonferroni *t* statistics (Miller, 1966). The criterion for all significance statements was $p \leq 0.05$.

RESULTS

There were no significant age differences in protein concentrations of tissue sonicates used in binding assays or enzyme assays in any of the regions tested (data not shown).

Significant age differences were noted in B_{max} for [^{3}H]QNB in the cerebral cortex (Table 1), striatum, (Table 2), and hippocampus (Table 3), but not the cerebellum (Table 4). No age differences in any of the other muscarinic binding parameters (K_{QNB}, $K_{Hi\text{-}Carb}$, $K_{Lo\text{-}Carb}$, B_{Hi}) were noted. Significant differences between B_{max} values at 12 months compared with four months did not occur in any of the four brain regions assayed. However, in the cerebral cortex, B_{max} was 38% and 57% lower in 18- and 24-month old mice, respectively, as compared with four-month old mice. A reduction also was apparent in the striatum, where B_{max} was 29% lower in 24-month old mice compared with 4-month old mice. In

TABLE 1. EFFECT OF AGING ON MUSCARINIC RECEPTOR BINDING AND CHOLINE ACETYLTRANSFERASE IN THE C57BL/6J MOUSE CEREBRAL CORTEX

	Age in Months			
	4	*12*	*18*	*24*
Muscarinic receptor binding *(n = 7–8)*				
K_{QNB} (nM [^{3}H]QNB)	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01
B_{max} (fmoles/mg protein)	964 ± 27	877 ± 39	597 ± 31*	408 ± 10*
$K_{Hi\text{-}Carb}$ (μM carbamylcholine)	0.23 ± 0.03	0.28 ± 0.04	0.31 ± 0.08	0.28 ± 0.03
$K_{Lo\text{-}Carb}$ (μM carbamylcholine)	76 ± 2	73 ± 3	76 ± 3	78 ± 2
B_{Hi} (% of B_{max})	36 ± 3	39 ± 2	41 ± 3	36 ± 3
Choline Acetyltransferase *(n = 6)*				
K_m (mM choline chloride)	2.00 ± 0.1	1.75 ± 0.2	1.85 ± 0.2	1.83 ± 0.2
K_m (μm acetyl coenzyme A)	11.8 ± 0.9	12.1 ± 0.9	14.5 ± 0.9	12.4 ± 0.9
V_{max} (nmoles/hr/mg protein)[a]	33.2 ± 3	39.4 ± 4	51.0 ± 4*	61.4 ± 4*

*Significantly different from previous ages, $p \leq 0.05$

[a]V_{max} values were determined from Eadie Hofstee plots where the concentration of acetyl coenzyme A was varied, and were not significantly different from values obtained by varying the choline chloride concentration.

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contrast, B_{max} was 11% greater in the hippocampus from 24-month old mice than in the hippocampus of 4-month old mice.

Values of K_m for ChAT did not vary with age in any brain region, nor did V_{max} in the cerebellum (Tables 1–4). Although there were no differences between V_{max} values obtained in 4-month old mice compared with those in 12-month old mice; V_{max} at 24-months was 36% higher in the hippocampus and 89% higher in the striatum, as compared with

TABLE 2. EFFECT OF AGING ON MUSCARINIC RECEPTOR BINDING AND CHOLINE ACETYLTRANSFERASE IN THE C57BL/6J MOUSE STRIATUM

	Age in Months			
	4	*12*	*18*	*24*
Muscarinic receptor binding *(n = 7–8)*				
K_{QNB} (nM [^{3}H]QNB)	0.09 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
B_{max} (fmoles/mg protein)	820 ± 45	702 ± 36	785 ± 28	583 ± 11*
$K_{Hi\text{-}Carb}$ (μM carbamylcholine)	0.62 ± 0.03	0.50 ± 0.08	0.49 ± 0.08	0.62 ± 0.08
$K_{Lo\text{-}Carb}$ (μM carbamylcholine)	97 ± 8	95 ± 5	117 ± 13	100 ± 8
B_{Hi} (% of B_{max})	40 ± 2	47 ± 3	48 ± 2	41 ± 12
Choline Acetyltransferase *(n = 6)*				
K_m (mM choline chloride)	1.73 ± 0.2	1.68 ± 0.2	1.80 ± 0.1	1.75 ± 0.1
K_m (μm acetyl coenzyme A)	13 ± 3	12 ± 4	15 ± 4	11 ± 4
V_{max} (nmoles/hr/mg protein)[a]	252 ± 31	264 ± 18	282 ± 47	477 ± 41*

*Significantly different from previous ages, $p \leq 0.05$

[a]V_{max} values were determined from Eadie Hofstee plots where the concentration of acetyl coenzyme A was varied, and were not significantly different from values obtained by varying the choline chloride concentration.

TABLE 3. EFFECT OF AGING ON MUSCARINIC RECEPTOR BINDING AND CHOLINE ACETYLTRANSFERASE IN THE C57BL/6J MOUSE HIPPOCAMPUS

	Age in Months			
	4	*12*	*18*	*24*
Muscarinic receptor binding *(n = 7–8)*				
K_{QNB} (nM [^{3}H]QNB)	0.08 ± 0.01	0.10 ± 0.02	0.09 ± 0.01	0.08 ± 0.01
B_{max} (fmoles/mg protein)	506 ± 8	485 ± 22	485 ± 24	565 ± 14*
$K_{Hi\text{-}Carb}$ (μM carbamylcholine)	0.17 ± 0.03	0.16 ± 0.03	0.13 ± 0.03	0.13 ± 0.03
$K_{Lo\text{-}Carb}$ (μM carbamylcholine)	70 ± 3	68 ± 9	68 ± 4	67 ± 3
B_{Hi} (% of B_{max})	21 ± 3	25 ± 2	21 ± 3	20 ± 4
Choline Acetyltransferase *(n = 7)*				
K_m (mM choline chloride)	1.50 ± 0.03	1.47 ± 0.12	1.53 ± 0.02	1.47 ± 0.08
K_m (μm acetyl coenzyme A)	9.44 ± 1.4	11.3 ± 1.4	10.2 ± 1.5	9.7 ± 1.1
V_{max} (nmoles/hr/mg protein)[a]	71.6 ± 5	70.0 ± 3	70.2 ± 4	97.6 ± 2*

*Significantly different from previous ages, $p \leq 0.05$

[a]V_{max} values were determined from Eadie Hofstee plots where the concentration of acetyl coenzyme A was varied, and were not significantly different from values obtained by varying the choline chloride concentration.

4-month old mice. In the cortex, V_{max} was 54% and 84% higher in 18- and 24-month old mice, respectively, than in 4-month old mice. Values of V_{max} obtained from Eadie-Hofstee plots where acetyl CoA concentrations were varied were almost identical to those obtained from Eadie-Hofstee plots where choline chloride concentrations were varied; in all cases, differences were less than 4% (data not shown).

TABLE 4. EFFECT OF AGING ON MUSCARINIC RECEPTOR BINDING AND CHOLINE ACETYLTRANSFERASE IN THE C57BL/6J MOUSE CEREBELLUM

	Age in Months			
	4	*12*	*18*	*24*
Muscarinic receptor binding *(n = 7–8)*				
K_{QNB} (nM [^{3}H]QNB)	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.02
B_{max} (fmoles/mg protein)	653 ± 32	599 ± 43	670 ± 32	690 ± 49
$K_{Hi\text{-}Carb}$ (μM carbamylcholine)	0.48 ± 0.04	0.47 ± 0.3	0.45 ± 0.03	0.44 ± 0.02
$K_{Lo\text{-}Carb}$ (μM carbamylcholine)	70 ± 3	65 ± 8	65 ± 8	69 ± 5
B_{Hi} (% of B_{max})	30 ± 4	26 ± 3	26 ± 2	32 ± 3
Choline Acetyltransferase *(n = 6)*				
K_m (mM choline chloride)	1.69 ± 0.03	1.72 ± 0.03	1.72 ± 0.10	1.72 ± 0.11
K_m (μm acetyl coenzyme A)	11.9 ± 3.1	12.1 ± 3.2	13.1 ± 1.9	11.9 ± 3.8
V_{max} (nmoles/hr/mg protein)[a]	12.9 ± 0.8	12.1 ± 1.2	12.0 ± 0.8	11.3 ± 0.8

*Significantly different from previous ages, $p \leq 0.05$

[a]V_{max} values were determined from Eadie Hofstee plots where the concentration of acetyl coenzyme A was varied, and were not significantly different from values obtained by varying the choline chloride concentration.

DISCUSSION

The present findings indicate that age effects on muscarinic receptor parameters in the brains of C57BL/6J mice are limited to changes in receptor densities. This conclusion is evident from the observations that three brain regions from 24-month old mice showed different values of B_{max} for [^{3}H]QNB compared with regions from younger mice, while no age differences were seen in K_{QNB}, $K_{Hi\text{-}Carb}$, $K_{Lo\text{-}Carb}$, or in the relative proportions of high- and lower-affinity agonist binding sites. These results agree generally with those of Freund (1980), who assayed [^{3}H]QNB binding in preparations of whole brains without cerebella from female C57BL/6J mice at 5-, 12-, 18-, 26- and 30-months of age. Freund noted that receptor densities in samples from 5-, 12-, and 18-month old mice did not differ, but were lower in samples from 26- and 30-month old mice. He also observed no age differences in receptor affinity. The results of the present study differ slightly from those of Strong *et al.* (1980), who studied male C57BL/6J mice, as we did. While both reports indicated age-associated declines in muscarinic receptor densities in the cortex and striatum, Strong *et al.* (1980) noted no age difference in the hippocampus, and we observed a significantly elevated receptor density in the aged hippocampus. The reason for the discrepancy between these studies is not apparent.

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Information also is available regarding age differences in muscarinic binding in the rat brain. Some of these age differences vary from our findings in mice. Therefore, it appears that age effects on central muscarinic receptors are species-specific. For example, whereas we report a significant elevation in hippocampal muscarinic binding site concentrations in C57BL/6J mice, an age-related decline was reported in Fischer-344 rats (Lippa *et al.* 1980), and no age differences were observed in Sprague Dawley and Long Evans rats (Strong *et al.*, 1980; Morin and Wasterlain, 1980). The present finding of decreased muscarinic binding in the cerebral cortex of old C57BL/6J mice is imilar to the age-associated decrease in the cortex of Sprague Dawley rats (Strong *et al.*, 1980; Morin and Wasterlain, 1980). Finally, studies of [^{3}H]QNB binding in Sprague Dawley rats (Strong *et al.*, 1980), and Long Evans rats, (Morin and Wasterlain 1980), as well as in C57BL/6J mice (this study) all demonstrated significantly lower values of B_{max} for [^{3}H]QNB in the striata of aged animals compared to younger animals. However, an age-related reduction in the affinity of [^{3}H]QNB for the binding site was noted in Sprague Dawley rats (Strong *et al.*, 1980) and not in studies of Long Evans rats (Morin and Wasterlain, 1980) or in C57BL/6J mice. It appears, therefore, that age effects on central muscarinic binding sites are not only species-specific, but also strain-specific.

In man, an age-related reduction in muscarinic receptor binding has been reported by some investigators (White *et al.*, 1977; Perry, 1980; Nordberg and Winblad, 1981), but not by others (Davies and Verth, 1978). We are aware of only one study of age effects on affinities of muscarinic receptors in human brain. This is the work of Nordberg and Winblad (1981), who reported that age effects on hippocampal muscarinic receptor parameters are limited to changes in receptor densities, and not in agonist affinities or in the proportion of high- to lower-affinity agonist binding sites. Considering these data, which are at variance with observations obtained in rats and mice, it is apparent that no rodent model examined to date faithfully mimics the conditions in the aged human brain.

In addition to alterations in muscarinic receptor densities, the present study also demonstrated substantial age effects on ChAT, the presynaptic acetylcholine synthetizing enzyme, in the cortex, hippocampus, and striatum, but not in the cerebellum, of the C57BL/6J mouse. Furthermore, all age effects on ChAT were due to an increased V_{max}, as

the affinities for acetyl CoA and choline chloride did not change with age in different brain regions. Although the kinetic properties of ChAT as a function of age in C57BL/6J mice have not been studied previously, ChAT activity at fixed substrate concentrations has been studied in mice of different ages (Vijayan, 1977; Strong *et al.*, 1980). Data from these studies are comparable to the present findings as all age-related changes in ChAT in the present study reflected changes in enzyme quantity (V_{max}), and saturating substrate concentrations were used in the previous studies. The age-related increase in V_{max} of ChAT in mice reported here agrees with our earlier findings regarding the regional activity of this enzyme (Waller *et al.*, 1983), but not with other earlier studies of C57BL/6J mice (Vijayan, 1977; Strong *et al.*, (1980). Vijayan (1977) reported no difference in V_{max} for ChAT in the aged (24 months) C57BL/6J mouse hippocampus compared with the activity in the young (8 months) hippocampus; however, we report a 36% increase in the V_{max} for ChAT in the aged (18 months) hippocampus compared with three to four month old hippocampus. Also in contrast to the increased V_{max} values reported here, Strong *et al.*, (1980) reported no age differences in cortical or hippocampal ChAT activities, and a decline in striatal ChAT activity in aged mice. The reason for these discrepancies is not known.

Discrepancies between the senescence-associated increases in ChAT reported here with findings in rat and human brain again demonstrate the species-dependence of age-related differences. For example, the V_{max} of ChAT was reduced in the cortex, cerebellum, and optic regions of aged Wistar rats while K_m values for both acetyl CoA and choline chloride were elevated in these regions (Mohan and Radha, 1978). Although age-related reductions in ChAT activities were observed in the cortices and striata of Fischer-344 rats (Strong *et al.*, 1980) and cerebella of Long-Evans hooded rats (Morin and Wasterlain, 1980), activities in hippocampi from Fischer-344 rats (Strong *et al.*, 1980) and Sprague Dawley rats (Lippa *et al.*, 1980) and in striata of Long Evans rats (Morin and Wasterlain, 1980) did not show age differences. In the human brain, age-related decreases in ChAT were observed in the hippocampus, striatum, and cortex (McGeer and McGeer, 1975; Perry *et al.*, 1977). These inconsistencies in age effects on ChAT in human brain as compared with mouse and rat brains further support the view that extrapolations from data obtained in rodents to generalizations about senesence of the human brain may be inaccurate.

Because the ChAT and muscarinic receptor assays in the present study were performed on tissues from different C57BL/6J mice, direct correlations between ChAT and muscarinic receptor data are not possible. However, it is interesting that whenever there was an age-related change in the quantity of ChAT, a presynaptic cholinergic marker, there was always an age-related change in the total muscarinic receptor density. This relationship was most dramatic in the cortex where the V_{max} for ChAT and B_{max} for muscarinic receptor binding were significantly different in both 18- and 24-month old mice compared with values at all preceding ages. These observations suggest that age effects on muscarinic receptors and ChAT are somehow linked.

The relationship between ChAT and muscarinic binding as a function of age varied with the brain region assayed. Although the effects of altered quantities of ChAT and muscarinic binding sites on the synthesis, storage, and release of acetylcholine as well as on postsynaptic events are not known, we suggest that in some cases the age effects on ChAT and total muscarinic binding may represent compensatory changes to maintain a critical level of cholinergic neurotransmission. For example, the elevation of ChAT as well as total concentration of muscarinic binding sites in the aged hippocampus may reflect an enhanced level of cholinergic neurotransmission to balance some as yet

undescribed deficit, that may be non-cholinergic. Conversely, the lack of age differences in ChAT and muscarinic binding in the cerebellum is not surprising in view of the dubious role of the cholinergic system in this brain region (Silver, 1974), and is in keeping with the speculation that age changes in cholinergic markers reflect compensation to maintain essential cholinergic neurotransmission. Still another situation occurred in the cerebral cortex and striatum where total muscarinic binding sites were lower but ChAT activity was higher in aged animals compared to younger animals. Presumably a signal from a postsynaptic neuron, which is receiving decreased cholinergic stimulation due to receptor losses, could be transmitted to the presynaptic neuron which, in turn, would synthesize more ChAT. Such an explanation has been offered for the accelerated formation of norepinephrine from tyrosine in animals treated with drugs that block adrenergic receptors (Snyder, 1976). Alternatively age-related decreases in muscarinic receptors may be an adaptation to limit cholinergic stimulation despite enhanced acetylcholine synthesis. In this regard, decreases in the density of cholinergic receptors have been noted in conditions of excessive cholinergic stimulation (Schiller, 1979; Uchida *et al.*, 1979; Ehlert *et al.*, 1980).

The present results clearly demonstrate specific regional age differences in central cholinergic systems. In some cases, these differences may reflect presynaptic compensatory mechanisms acting to maintain critical levels of cholinergic function.

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DIFFERENTIAL EFFECTS OF DIETARY CALORIC AND PROTEIN RESTRICTION IN THE AGING RAT[1]

TERESA A. DAVIS[2], CONNIE W. BALES[3], and ROY E. BEAUCHENE[4]

Tennessee Agricultural Experiment Station and Department of Nutrition and Food Sciences
229 College of Home Economics, The University of Tennessee, Knoxville, TN 37996-1900

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Abstract—Numerous studies have shown caloric restriction retards the physiological decline and increases the life span of animals. However, in these studies protein consumption was also reduced; thus, whether the beneficial effects were due to caloric or to protein restriction is unclear. To examine independently the effects of caloric and protein restriction on growth, renal function, and survival, caloric restricted male rats were fed 18, 30 or 42 percent casein diets that provided two-thirds of the quantity of diet consumed by groups fed 12, 20, or 28 percent casein diets *ad libitum*, respectively. Hence, caloric restricted groups consumed the same amount of protein as their paired *ad libitum* fed groups but one-third fewer calories. The results showed that caloric restriction decreased mature body weight, increased the rate of attaining mature body weight, retarded the age-associated decline in renal function, and increased survival. Protein restriction had no effect on mature body weight, decreased maturation rate, improved renal function, and decreased survival. Thus, protein restriction did not contribute to the survival-promoting effects of caloric restriction in rats.

INTRODUCTION

THE LIFE span of experimental animals has been increased by nutritional modification, particularly by caloric restriction (Weindruch *et al.*, 1982; Beauchene *et al.*, 1979; Berg and Simms, 1960; McCay *et al.*, 1939). This increased longevity may have been due, in part, to a restriction in dietary protein since in almost all studies, calorie restriction was accompanied by a parallel reduction in protein intake (Beauchene *et al.*, 1979; Berg and Simms, 1960; McCay *et al.*, 1939). However, data available on the effect of dietary protein on life span are conflicting. Divergent results have been reported (Leto *et al.*, 1976; Miller and Payne, 1968; Nakagawa *et al.*, 1974; Ross and Bras, 1973; Ross and Bras,

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[2]Present address: Washington University School of Medicine, Department of Preventive Medicine, 4566 Scott Avenue, St. Louis, MO 63110.

[3]Present Address: University of Texas at Austin, Department of Home Economics, Graduate Nutrition Division, Austin, TX 78712.

[4]Address reprint requests to third author at: Department of Nutrition and Food Sciences, 229 College of Home Economics, The University of Tennessee, Knoxville, TN 37996-1900.

1975) and may be due to the large variation in the levels of dietary protein utilized and the differences in caloric intake.

Restriction of caloric and/or protein intake of experimental animals decreased their body weight (Goodrick, 1978; Berg and Simms, 1960), reduced the activities of certain enzymes (Leto *et al.*, 1976), improved immune responses (Weindruch *et al.*, 1979), and delayed the development of diabetes (Gerritsen, 1976). In addition, dietary restriction delayed the age-associated decline in renal function (Adams and Barrows, 1973; Beauchene *et al.*, 1965; Saxton and Kimball, 1941), decreased the incidence of renal lesions (Bras and Ross, 1966), and reduced age-associated changes in renal morphology (Johnson and Barrows, 1980). In our laboratory, changes in renal function during hypocaloric feeding were quantified as reductions in urinary protein excretion and improved renal transport of para-aminohippuric acid (Tucker *et al.*, 1976).

The present study was designed to examine the independent effects of caloric and protein restriction on aging. Thus, the effects of caloric restriction with or without protein restriction, and of protein restriction with or without caloric restriction were determined on survival, renal function, and growth of rats. The results indicated that survival and renal function were more sensitive to restriction of caloric intake than to that of dietary protein.

METHODS

Animals and diet

Male Wistar rats were obtained as weanlings (National Research Laboratories) and individually housed in hanging wire cages. Animals were provided water *ad libitum* and fed a nutritionally complete 20 percent casein diet (Table 1) *ad libitum* until 32 days of age when they were placed on experimental diets. At the beginning, 36 animals (old) were assigned to each of six dietary treatments and maintained for two years (Table 2). Twelve months later, 12 additional 32-day old rats (young) were assigned and maintained for one year on each of the six dietary treatments. The restricted groups (R) were fed 18, 30, and 42 percent casein diets that provided two-thirds of the mean quantity of diet consumed by groups fed 12, 20, and 28 percent casein diets *ad libitum* (A), respectively. Thus, R groups consumed the same quantity of protein as their paired A groups but one-third less calories. Diets of different protein levels were prepared by substituting amounts of 1:1 mixture of cornstarch and sugar for casein.

Daily feed intakes of restricted groups were based on average intakes of ad libitum-fed animals established over seven-day periods; all intakes were corrected for spillage. Daily feed intakes were measured throughout the first year of life and every other week thereafter. Rats were weighed weekly until six months of age, biweekly until 12 months and monthly thereafter.

Growth curves

The mature body weight in grams and the rate of attaining mature body weight were calculated from 16 body weights obtained at intervals for each animal during its first year of life. The equation used (Brody, 1964) was as follows: $\mathbf{W} = \mathbf{A} - \mathbf{B}e^{-\mathbf{k}t}$ where $\mathbf{W}$ = animal weight in grams at a given age, $\mathbf{A}$ = mature body weight in grams, $\mathbf{B}$ = integration constant, $\mathbf{e}$ = base of natural logarithms, $\mathbf{k}$ = rate of growth with respect to that yet to be made, and $\mathbf{t}$ = age in weeks. Rats with larger $\mathbf{k}$ values reach mature body weights more rapidly. Values of $\mathbf{A}$, $\mathbf{B}$, and $\mathbf{k}$ were computed for each rat by the Gauss-Newton method (Bard, 1974), and a non-linear least square procedure available in SAS (Barr and Goodnight, 1979). From these individual data, mean values of $\mathbf{A}$, $\mathbf{B}$, and $\mathbf{k}$ for each experimental group were calculated and used to construct growth curves.

Renal determinations

Within one month prior to sacrifice, each animal was placed in a metabolism cage for 72 hours. Urine was collected daily and centrifuged (2,000 rpm × 15 mintues). Protein was measured in the supernatant using the biuret reagent (Saifer and Gerstenfeld, 1964). Rats were stunned by a blow to the head and decapitated. Kidneys were removed, chilled on ice, decapsulated, and weighed. Right kidneys were sliced with a Stadie-Riggs microtome

fitted with a 0.5 mm head; four slices were used to determine para-aminohippuric acid (PAH) transport (Adams and Barrows, 1963).

Survival

Cages were checked daily for dead animals. Percent survival was calculated at regular intervals until the rats were two years of age at which time all survivors were killed.

Statistical analyses

Data were analyzed using analysis of variance (Snedecor and Cochran, 1967) to test for differences among dietary treatments and ages. Specific effects of dietary protein and caloric levels on each parameter were tested using orthogonal comparisons. If there were no significant interactions between dietary protein and caloric levels, the effect of protein was tested by pooling the variances of the *ad libitum* and restricted groups with the same protein intake. A Chi-square test was used for differences in survival rates among groups. Probability levels of less than 0.05 were considered statistically significant.

RESULTS

Growth and feed intake

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The growth curves of all groups were similar in shape, but the A groups were significantly higher than those of the R groups (Figure 1). Also, as shown in Table 3, the mean predicted mature body weights of A were 47% greater than those of R groups ($p < 0.0001$). However, rates of attaining mature body weights (k values) were 7% greater in R than in A groups ($p < 0.005$). The dietary protein levels of either the A or the

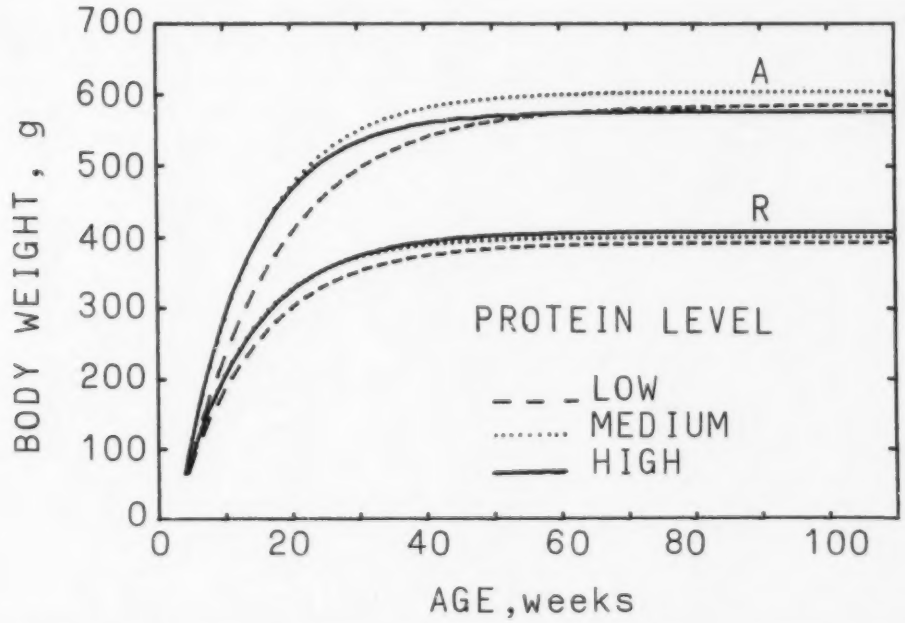

FIG. 1. Influence of level of dietary protein on calculated growth curves (Brody, 1964) of rats fed *ad libitum* (A) and restricted (R) diets.

TABLE 1. COMPOSITION OF 20% CASEIN DIET

Dietary Component	*Percent of Diet*
Casein, High nitrogen[a,b]	20.0
Sucrose	29.0
Cornstarch	29.0
Crisco	6.0
Wesson oil	2.0
Vitamin Mix[a,c]	2.0
Salt Mix[a,d]	3.0
Alphacel[a]	9.0

[a]ICN Nutritional Biochemicals, Cleveland, Ohio 44128.

[b]Determined by the Kjeldahl method to contain 91.5% protein.

[c]Vitamin Diet Fortification Mixture formulated to supply the following amounts of vitamins (g/kg vitamin mix): vitamin A, 4.5; vitamin D, 0.25; thiamin hydrocholoride, 1.0; riboflavin, 1.0; niacin, 4.5; p-aminobenzoic acid, 5.0; calcium pantothenate, 3.0; pyridoxine hydrochloride, 1.0; ascorbic acid, 45.0; inositol, 5.0; choline choride, 75.0; menadione, 2.25; biotin, 0.02; folic acid, 0.09; vitamin B_{12}, 0.00135; alpha-tocopherol, 5.0; and sufficient dextrose to make 1 kg.

[d]Hubbell, R.B., Mendel, L.B. & Wakeman, A.J. (1937). Salt mix formulated to supply the following amounts of minerals (g/kg salt mixture): $CaCO_3$, 543.0; $MgCO_3$, 25.0; $MgSO_4$, 16.0; NaCl, 69.0; KCl, 112.0; KH_2PO_4, 212.0; $FePO_4 \cdot 4H_2O$, 20.5; KI, 0.08; $MnSO_4$, 0.35; NaF, 1.00; $Al_2(SO_4)_2K_2SO_4$, 0.17; and $CuSO_4$, 0.90.

R groups did not affect predicted mature body weights. However, low dietary protein levels at both caloric levels (A and R) resulted in 19% lower k values ($p < 0.0005$) than those obtained for rats fed medium or high levels of protein.

The feed intakes of A groups are shown in Figure 2 (R groups were fed two-thirds of the amount of feed consumed by their paired A groups). The intakes of animals on the low protein diet were 5% less ($p < 0.05$) than those on medium or high levels of protein, which were not significantly different. Feed intakes were age-dependent, peaking at four months of age and decreasing ($p < 0.001$) slightly thereafter.

TABLE 2. DIETARY TREATMENTS[a]

Dietary protein Level	*% Casein in Diet*	
	Ad libitum	*Restricted*[b]
Low	12	18
Medium	20	30
High	28	42

[a]Initially 48 animals per dietary treatment, 12 designated "young," 36 as "old."

[b]Restricted animals were fed the same amount of protein but one-third fewer calories than their ad libitum counterparts.

TABLE 3. BODY GROWTH AS A FUNCTION OF DIETARY PROTEIN LEVEL OF AD LIBITUM-FED AND RESTRICTED RATS

Dietary Group	*Number of Animals*	*Predicted mature Body weight*[a,b] *(Grams)*	*Rate of Attaining mature Body weight*[c,d]
Ad libitum			
Low protein	36	575 ± 13.1	0.0686 ± 0.00191
Medium protein	36	608 ± 13.0	0.0864 ± 0.00351
High protein	36	569 ± 11.6	0.0956 ± 0.00317
Restricted			
Low protein	36	389 ± 4.04	0.0810 ± 0.00215
Medium protein	36	397 ± 4.02	0.0967 ± 0.00176
High protein	36	404 ± 3.74	0.0904 ± 0.00176

[a]A in Brody (1964) equation.
[b]*Ad libitum* greater than restricted ($p < 0.0001$).
[c]k in Brody (1964) equation.
[d]*Ad libitum* less than restricted ($p < 0.005$); directly related to level of dietary protein (orthogonal comparison; $p < 0.0005$).

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Renal responses

Kidney weights were smaller in caloric restricted young (18%, $p < 0.05$) and old (41%, $p < 0.0001$) rats as compared to their *ad libitum* fed controls as shown in Table 4. When expressed as a percent of body weight, kidney weights were 18% greater in young R than in young A ($p < 0.05$). Kidney weights in grams or as percent of body weight were unaffected by the dietary protein level. Old rats had higher ($p < 0.001$) kidney weights than young animals when expressed in grams (26%) or as a ratio to total body weight (28%).

PAH transport, a measure of the kidney's ability to perform osmotic work (Adams and Barrows, 1963), 25% less ($p < 0.07$) in old A than in old R (Table 5). Urinary protein excretion was 250% greater in old A than in old R rats ($p < 0.0001$). PAH transport was unaffected by dietary protein level, but urinary protein excretion increased as dietary protein increased in old rats ($p < 0.05$). As compared to young rats, old animals decreased PAH transport 39% ($p < 0.001$) and increased urinary protein excretion 118% ($p < 0.001$). PAH transport and urinary protein excretion were inversely correlated in old A rats fed medium ($r = -0.92$, $p < 0.01$) or high ($r = -0.90$, $p < 0.01$) protein diets.

Survival

Survival data for all groups are presented in Figure 3 and Table 6. At two years of age (104 weeks) restricted rats fed high, medium and low levels of dietary protein had survival values of 53%, 61% and 31%, respectively; corresponding values for *ad libitum* fed rats were 33%, 31% and 19%. Survival values for restricted rats were significantly higher than those of *ad libitum* fed rats ($p < 0.0005$). Low levels of dietary protein resulted in a decrease in survival in both *ad libitum* fed and restricted rats ($p < 0.05$). The highest survival rate was observed in R animals fed the medium level of dietary protein.

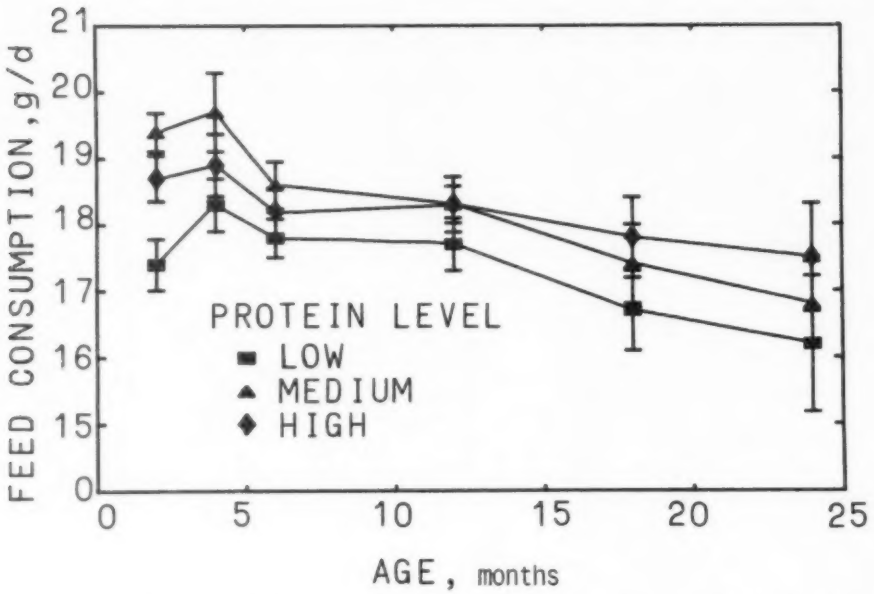

FIG. 2. Feed intake of *ad libitum* fed rats as a function of dietary protein level and age. Values plotted are mean intakes per day per rat ± SEM.

TABLE 4. INFLUENCE OF LEVEL OF DIETARY PROTEIN AND AGE ON KIDNEY WEIGHTS[a] OF *AD LIBITUM* AND RESTRICTED RATS

	Kidney wt[b]		$\frac{\text{Kidney wt}}{\text{Body wt}} \times 100$[b]	
Dietary group	*Young*[c]	*Old*[d]	*Young*[e]	*Old*
	g		%	
Ad libitum				
Low protein	2.51 ± 0.118 (8)[f]	3.54 ± 0.388 (5)	0.498 ± 0.0142 (8)	0.648 ± 0.0821 (5)
Medium protein	2.59 ± 0.098 (9)	3.67 ± 0.802 (5)	0.504 ± 0.0268 (9)	0.742 ± 0.212 (5)
High protein	2.89 ± 0.148 (8)	4.33 ± 0.621 (7)	0.553 ± 0.0282 (8)	0.785 ± 0.114 (7)
Restricted				
Low protein	2.23 ± 0.051 (9)	2.17 ± 0.211 (5)	0.623 ± 0.0141 (9)	0.708 ± 0.0523 (5)
Medium protein	1.89 ± 0.043 (10)	2.22 ± 0.069 (8)	0.574 ± 0.0239 (10)	0.771 ± 0.0377 (8)
High protein	2.46 ± 0.079 (11)	2.43 ± 0.047 (7)	0.639 ± 0.0201 (11)	0.696 ± 0.0254 (7)

[a]Weights of right plus left kidney.
[b]Old greater than young ($p < 0.001$).
[c]*Ad libitum* greater than restricted ($p < 0.05$).
[d]*Ad libitum* greater than restricted ($p < 0.0001$).
[e]*Ad libitum* less than restricted ($p < 0.05$).
[f]Number of rats per group in parentheses.

TABLE 5. URINARY PROTEIN EXCRETION IN MALE RATS AS A FUNCTION OF DIET AND AGE[a]

Dietary group	PAH[b] (μg/g)		Urinary protein[b] (mg/day)	
	Young	Old[c]	Young	Old[d,e]
Ad libitum				
Low protein	211 ± 9.86	95 ± 32.1	10.8 ± 1.03	31.3 ± 18.1
Medium protein	218 ± 19.9	106 ± 45.1	16.9 ± 3.82	81.2 ± 31.1
High protein	184 ± 25.0	98 ± 22.7	23.5 ± 2.19	46.2 ± 14.8
Restricted				
Low protein	186 ± 13.7	139 ± 27.0	10.3 ± 1.76	14.4 ± 4.13
Medium protein	185 ± 20.3	125 ± 22.9	9.5 ± 0.94	13.5 ± 1.49
High protein	171 ± 17.3	135 ± 22.3	22.7 ± 3.33	17.3 ± 3.79

[a]Number of rats per group ranged from 5 to 11.
[b]Old vs. young ($p < 0.001$).
[c]Ad libitum vs. restricted ($p < 0.07$).
[d]Ad libitum vs restricted ($p < 0.0001$).
[e]Directly associated with dietary protein level (orthogonal comparison; $p < 0.05$).

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DISCUSSION

The results of this study demonstrate that rats fed calorically restricted diets (67% of *ad libitum* fed controls) survived longer than those fed *ad libitum* even when both caloric groups consumed the same amount of protein. In previous studies (Beauchene *et al.*, 1979; McCay *et al.*, 1939; Miller and Payne, 1968; Ross and Bras, 1973; Ross and Bras, 1975), reduction in caloric intake was accompanied by a proportional decrease in protein intake, thus making it difficult to determine whether the increase in survival was related primarily to caloric or to protein restriction. Although caloric intake had a greater effect

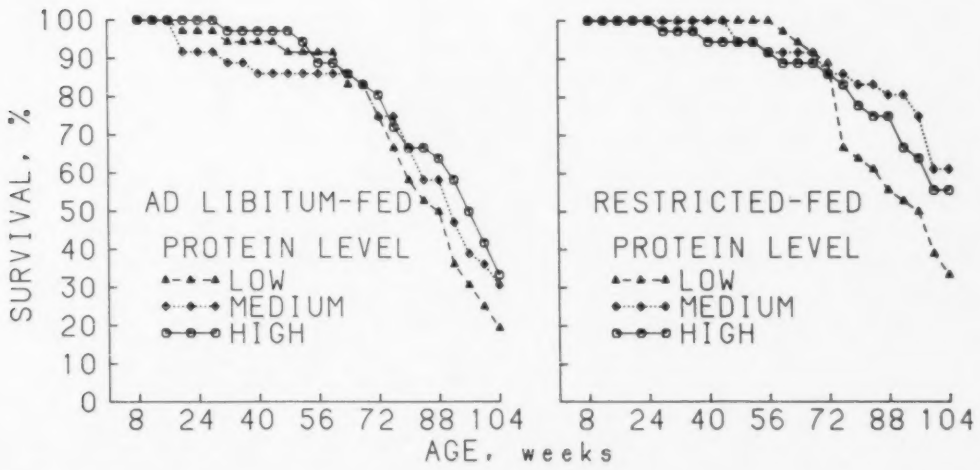

FIG. 3. Influence of protein and caloric restriction on survival of rats (n = 36 per dietary treatment initially).

TABLE 6. SURVIVAL RATES OF RATS FED *AD LIBITUM* AND RESTRICTED DIETS

	Percent Survival After	
Dietary group	*1 year*	*2 years*[a,b]
Ad libitum		
Low Protein	97	19
Medium protein	86	31
High protein	94	33
Restricted		
Low protein	100	31
Medium protein	94	61
High protein	94	53

[a]Ad libitum vs. restricted ($p < 0.0005$).

[b]Directly associated with dietary protein level (orthogonal comparison; $p < 0.05$).

on survival than protein intake in the present study, low levels of dietary protein in both calorie-restricted and *ad libitum* fed groups were associated with decreased survival rates. While caloric restriction (33%) increased survival, the concomitant reduction in feed intake (5%) by both the *ad libitum* fed and restricted rats fed the low protein diets was associated with decreased survival.

Reductions in feed intake in animals fed low protein diets have been reported previously (Leto *et al.*, 1976; Ross *et al.*, 1970). Ross and Bras (1973) reported higher survival rates in rats fed high protein diets. Other studies have reported conflicting results but these utilized levels of protein that resulted in a stunting of growth (Goodrick, 1978; Leto *et al.*, 1976; Miller and Payne, 1968).

Higher rates of attaining mature body weight (k values) may be related to increased survival. Although caloric restriction increased survival and decreased body weight, k values of restricted rats were greater than those *ad libitum* fed. In both A and R groups, however, a low level of dietary protein resulted in decreased survival and less rapid growth rates. In contrast to our findings, Goodrick (1978) reported that a reduction in growth rate and a longer growth duration enhanced survival. Growth rates are not expressed in the same terms in the present study and that of Goodrick, however.

Dietary factors which prolong survival may vary in their effects on renal function. For example, caloric restriction increased percent survival and also improved renal function. On the other hand, protein restriction decreased survival and yet improved renal function. Thus, it appears that while high levels of dietary protein can adversely affect the function of a specific biological system, that is, the kidney, moderately high protein diets are beneficial for other systems in these animals, and therefore, favorably influence their survival.

Renal function and survival appear to be more sensitive to the calories consumed than to the quantity of dietary protein ingested. While caloric restriction improved renal function, low levels of dietary protein decreased urinary protein excretion that becomes elevated with age, but did not significantly affect renal PAH transport. However, these two measures of renal function were inversely correlated in old *ad libitum* fed animals.

Survival was enhanced to a greater extent by caloric restriction than by increasing the level of protein in the diet.

SUMMARY

The independent effects of caloric and protein restriction on survival, growth, and renal function were assessed in aging adult male rats fed for up to two years on various diets. Restricted groups were fed 18, 30, and 42 percent casein that provided two-thirds of the quantity of diet consumed by groups fed 12, 20 and 28 percent casein *ad libitum*, respectively. Thus, the restricted groups consumed the same amount of protein as their paired *ad libitum* fed groups but one-third fewer calories.

Calorie restriction decreased the mature body weight but increased the rate of attaining mature body weight. Also, the age-associated decline in renal function was improved by calorie restriction as indicated by *in vitro* transport of para-aminohippuric acid and excretion of urinary protein. Finally, the percent survival at two years was significantly increased by calorie restriction.

Low levels of dietary protein had no effect on mature body weight but decreased the rate of maturation. Also, dietary protein restriction improved renal function, but unlike caloric restriction, decreased survival.

Caloric restriction was more effective than protein restriction in altering mature body weight, renal function, and survival of rats.

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AGE-DEPENDENT CHANGES OF PROTEIN STRUCTURE

The Properties of Young and Old Rabbit Aldolase Are Restored After Reversible Denaturation

ALEXANDER P. DEMCHENKO, NELLY N. ORLOVSKA* and ANATOLY G. SUKHOMUDRENKO**

Institute of Gerontology, Academy of Medical Sciences of the USSR, Kiev
*Palladin Institute of Biochemistry, Academy of Sciences of the Ukrainian SSR, Kiev
**Institute of Biological Physics, Academy of Sciences of the USSR, Pushchino, Moscow Region, USSR

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INTRODUCTION

THE PROBLEM OF age-dependent changes of protein structure and function is under active investigation (Rothstein, 1975, 1977; Klefenz and Zuckerman, 1978; Gershon *et al.*, 1979; Demchenko and Orlovska, 1981). The occurrence of altered protein molecules in aging organisms, their distribution in various organs, tissues and cells, as well as the properties of altered enzyme molecules are the principal areas of research.

The comparative study of highly purified preparations of young and old rabbit skeletal muscle aldolase (Orlovska *et al.*, 1980; Demchenko and Orlovska, 1980) revealed that despite the similarity in affinity and ion-exchange chromatographic and electrophoretic behavior, old muscle aldolase demonstrated significant differences in the specific activity and kinetic properties of the aldolase reaction. Besides, the conformational differences between old and young rabbit muscle aldolase were revealed and described by means UV difference spectroscopy, thermal perturbation difference spectra and UV fluorescence. Tyrosine and tryptophan residues of the old rabbit aldolase appear to be in less hydrophobic environment than those of the young one. Old rabbit aldolase conformation is more heat-resistant. However, the conformational transitions on substrate binding are found to be similar in aldolase of animals of both ages.

The present study is undertaken to elucidate whether the two age-dependent forms are conformational isomers (conformers) that differ only by the state of conformation and formation of secondary noncovalent bonding, or the alterations in primary (covalent) structure take place and are responsible for the observed differences in conformation and enzyme behavior. For this purpose, at the first step we performed the studies of aldolase

Correspondence address: Dr. A.P. Demchenko, Inst. of Gerontology AMS USSR, Vyshgorodskaya St. 67, 252555 Kiev-114, USSR

conformation in aging by the circular dichroism method. At the second step, we demonstrated that being unfolded by acid denaturation and reversibly folded under native conditions, aldolase conformation returns to its initial state, with age-dependent differences being restored.

MATERIALS AND METHODS

Aldolase preparations

Fructose-1,6-diphospate aldolase (EC 4.1.2.13) was isolated from skeletal muscles of the young (6–8 month) and old (52–70 month) rabbits and purified as described previously (Orlovska *et al.*, 1980). Short-term storage of protein preparations has been performed in saturated ammonium sulfate at 0–4°C. Protein concentration was determined by spectrophotometry, using the value $E^{1\%}_{280nm} = 9.1$.

Activity

Aldolase activity was determined spectrophotometrically. The NADH oxidation kinetics was observed at 340 nm in 0.05M Tris-HCl, pH 7.5, 0.5% NADH and 0.01M fructose-1,6-diphosphate in the presence of α-glycerophosphate dehydrogenase (Lytvynenko *et al.*, 1972).

Denaturation and renaturation conditions

Acid denaturation and renaturation at neutral pH was performed along the procedure of Engelhard *et al.* (1976) with some modifications. Stock solutions of aldolase were dialyzed in cellophane tubes at +4°C overnight against 0.2M potassium phosphate buffer, pH 7.6, containing 1mM EDTA and 0.1mM dithiothreitol. An aliquote of protein solution was separated, diluted 60-fold with the same buffer and further analyzed as initial "native enzyme." Another aliquote was diluted 4-fold with 1M glycine-H_3PO_4 buffer, pH 2.3 containing 1mM EDTA and 1mM dithiothreitol and incubated for 20–30 min at 20°C. Then it was separated into two aliquotes. One was diluted 15–fold with water and further analyzed as "acid-denatured protein." The other was renatured by 15-fold dilution with 0.2M K_2HPO_4 in the cold, the final pH being 7.3–7.6. After the incubation for 30 min and more at +4°C this preparation was analyzed as "renatured enzyme." This procedure resulted in almost complete restoration of enzyme structure and activity. The final protein concentration was of the order of 0.01 mg/ml and was essentially the same for all three preparations.

Circular dichroism spectra

The spectra of circular dichroism were recorded on dichrographs J-20 Jasco (Japan) and Mark-5 Jobin-Ivonne (France) in 1 mm silica cells. The α-helical content was calculated by the ellipticity $[\theta]_{209}$ and $[\theta]_{222}$ values. For 100% α-helices $[\theta]_{222}$ was accepted to be $35 \cdot 10^3$. The aminoacid residue mean molecular weight of aldolase was accepted to be 112. The assistance of Dr. A. Poletaev (The institute of Molecular Biology, USSR Academy of Sciences, Moscow) is acknowledged.

Fluorescence spectra

Fluorescence emission spectra in the spectral range 300–380 nm were recorded on Hitachi MPF-4 or Perkin Elmer MPF 44B fluorescence spectrophotometers at 20°C in rectangular 1 cm silica cells. Monochromator special slits were 2 nm (excitation) and 5 nm (emission), ratio operating mode, scan speed 30 nm/min. In the studies of the dependence of spectra on temperature, the temperature was set by ultrathermostat and measured directly in the cell by the thermistor.

RESULTS

The study of age-dependent differences of old and young rabbit adolase by circular dichroism

The circular dichroism spectra of young (a) and old (b) rabbit aldolase are presented in Figure 1. The spectra are numbered in accordance with different individual animals used as a source of aldolase preparations.

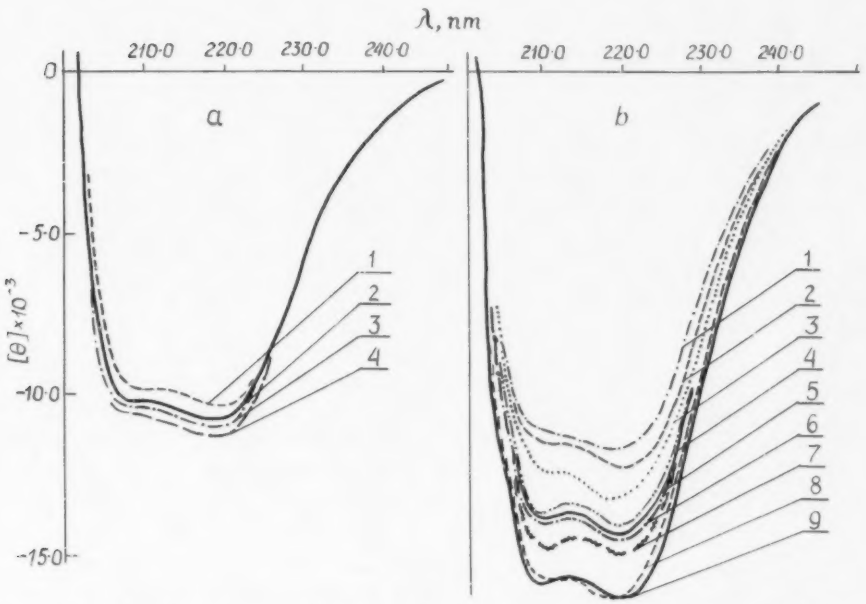

FIG. 1. The circular dichroism spectra of native young a) and old b) rabbit muscle aldolase in 0.2M potassium phosphate buffer, pH 7.4.

1–9 are the different animal's preparation numbers.

In the case of young rabbit aldolase the differences between the preparations studied are within the method's error range (5–8%) resulting from errors in determination of protein concentration and circular dichroism. The α-helical content of aldolase molecule determined for the spectra of Figure 1a is 26–28%, which is in agreement with the published values (Stellwagen and Schachman, 1962; Gerschitz *et al.*, 1977; Jaenicke, 1978).

The studies of old rabbit aldolase showed the dispersion of the circular dichroism spectra between preparations studied to be significantly greater (Figure 1b). Besides, the absolute value of circular dichroism intensity for all preparations is significantly greater, than in the case of young rabbit aldolase (Figure 1a). These results clearly indicate the increased helical content of old rabbit aldolase. For the preparation that corresponds to spectrum 1, the helical content is 34%, and for preparations 8 and 9 which are characterized by the highest circular dichroism intensity, the helical content reaches 42%.

The data presented indicate that under normal conditions in young animal organism the secondary structure of aldolase is characterized by moderate amount of α-helices. In aging organism the helical structure formation of formerly nonstructural part of protein polypeptide chain takes place. This may result in characteristic features of aldolase molecules in aging animals which had been observed earlier (Demchenko and Orlovska, 1980), that is, the alteration of the environment of tyrosine and tryptophan residues and the increase of the area of thermal stability.

Thus, the circular dichroism spectra are sensitive enough to detect significant conformational differences of aldolase in aging. This method can be used therefore to follow age-dependent conformational changes of this protein under different influences, specifically in denaturation and renaturation studies.

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The study of renaturation of old rabbit aldolase after acid denaturation

The restoration of the native protein properties after denaturation by various agents is an important property of rabbit muscle aldolase (Stellwagen and Schachman, 1962; Gerschitz *et al.*, 1977; Jaenicke, 1978). After acid denaturation and subsequent renaturation (incubation at neutral pH) up to 95% of the initial activity is recovered (Vimard *et al.*, 1975; Engelhard *et al.*, 1976; Gerschitz *et al.*, 1977). In this case the restoration of secondary, tertiary and quartenary structure of the enzyme may be followed by different methods (Engelhard *et al.*, 1976; Rudolph *et al.*, 1976). Under similar conditions we studied denaturation and renaturation of aldolase preparations from old and young animals. The methods of circular dichroism and UV fluorescence and the control of the recovery of activity were applied to follow the restoration of enzyme structure and function.

The circular dichroism spectra of different preparations of young and old rabbit aldolase under the conditions of acid denaturation (a) and after renaturation at the neutral pH (b) are presented in Figure 2. For the denatured proteins, almost complete disappearance of circular dichroism maxima 209 and 222 nm characteristic for the α-helices is observed. No age-dependent differences are revealed in the acid-denatured preparations of aldolase. The spectra of the renatured enzyme preparations show the same helical content as the initial native preparations. These results demonstrate that in the preparations of renatured aldolase of the old animals the high α-helical content of molecule which existed before the denaturation is restored. (Its estimated value is 38% for the preparation 2 and 42% for the preparation 4.) Such a high helical content is the peculiarity of initial native preparations of old rabbit aldolase (see Figure 1) and is not observed in the aldolase of young animals.

Figure 3 presents the results of a typical experiment of acid denaturation and renaturation of aldolase observed by the UV fluorescence spectra. The fluorescence spectra of the young and old rabbit aldolase were shown to differ both by the position of fluorescence spectra and by quantum efficiency (Demchenko and Orlovska, 1980). Both in the young and old rabbit aldolase the denaturation brings about the significant long-wave shift of the spectra reaching 350 nm, which is an indication of the refolding of the protein molecule and the transition of tryptophan residues, which are responsible for the fluorescence of that protein to water environment. The age-dependent differences in the spectra of the denatured proteins are not revealed. On renaturation the reversal shift of fluorescence spectra in the short-wave direction is observed. It reaches almost completely the position characteristic for the initial native enzyme. The age-dependent differences are restored.

The results of the experiment on the dependence of initial native and renatured aldolase conformation on temperature are presented in Figure 4. On the ordinate is the parameter I_{310}/I_{350} which is determined from the fluorescence spectra (the ratio of fluorescence intensity at 310 and 350 nm), which depends on the position of the spectrum on the wavelength scale and does not depend on fluorescence intensity (Zyma and Demchenko, 1976). On denaturation it decreases reflecting longwave shift of the spectrum, and on renaturation it increases. Figure 4 presents typical results for three different preparations of aldolase of old animals which show that the restoration of I_{310}/I_{350} is almost complete. Not only the characteristic features of the old rabbit enzyme are restored, but also the less prominent differences between its preparations that refer to different animals. The temperature-dependent I_{310}/I_{350} also shows that the renatured aldolase does not differ from the initial

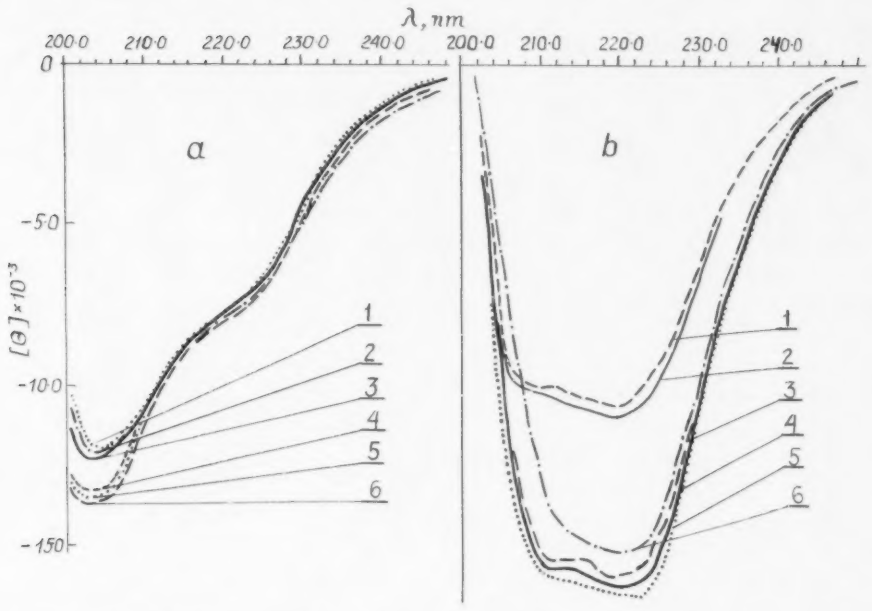

FIG. 2. The circular dichroism spectra of denatured and renatured muscle aldolase of young (1–2) and old (3–6) rabbits.

a) denatured protein in 1M glycine-phosphate buffer, pH 2.3.

b) protein after renaturation at pH 7.3–7.6

1–6 are the different animal's preparation numbers.

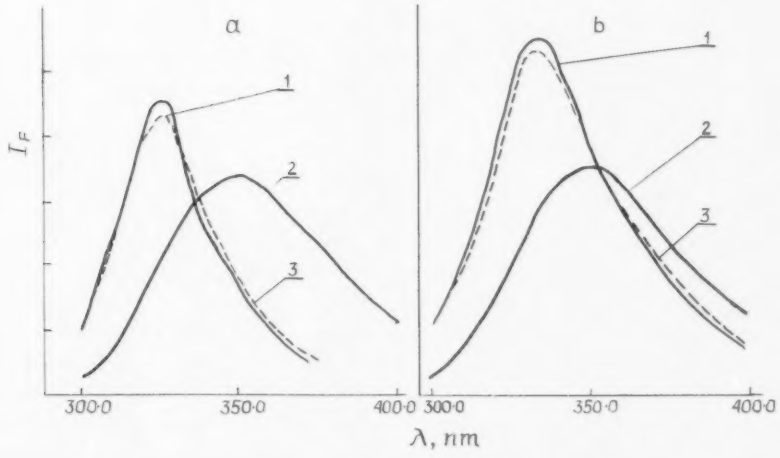

FIG. 3. The UV fluorescence spectra of young (a) and old (b) rabbit muscle aldolase.

1) native protein; 2) denatured at pH 2.3; 3) renatured protein. (Fluorescence excitation wavelength 295 nm.)

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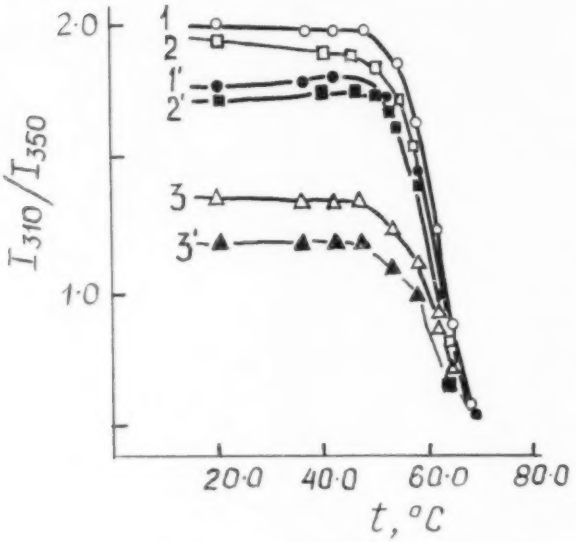

FIG. 4. The dependence of old rabbit muscle aldolase UV fluorescence parameter I_{310}/I_{350} on temperature. 1–3 are the different animals's preparation numbers; native enzyme 1'–3' are the same preparations, renatured enzyme. (Excitation wavelength 295 nm.)

native enzyme with respect to thermal stability of the protein conformation (Figure 4). Such temperature dependence significantly differs from that for young rabbit aldolase by the greater range of thermal stability (Demchenko and Orlovska, 1980).

On aldolase denaturation and renaturation the almost complete recovery of activity is observed (Table 1). The specific activity of old rabbit aldolase preparations is lower than that of the young and can be characterized by greater data scattering. The age-dependent difference in activity and the observed difference between individual preparations of the old rabbit aldolase is restored on renaturation.

Thus, the comparative analysis of aldolase preparations of the young and old rabbits before and after denaturation-renaturation shows the restoration of the initial protein conformation and activity. The renatured enzyme retains the age-dependent differences of these properties.

DISCUSSION

The numerous indirect data (Rothstein, 1975; 1977; Demchenko and Orlovska, 1981) as well as the results of the latest studies on purified protein preparations by structural methods (Sharma and Rothstein, 1978; Demchenko and Orlovska, 1980; Gafni, 1981) indicate the possibility of conformational changes of proteins in aging. The observed age-dependent differences in conformation were rather subtle. Therefore, the significant alterations of aldolase secondary structure observed in the present study were unexpected. Previously, the increase of α-helical content of α-crystallin in the central (senile) zone of the hen eye lens (Ju *et al.*, 1977) and the increased helicity of this protein in old animals compared to young ones (Horwitz and Platigorsky, 1980) were observed. At the same time, Schofield (1980) did not observe any change in albumin secondary structure in aging mice. The age-dependent changes of protein structure observed by circular dichroism in

TABLE 1. SPECIFIC ACTIVITY (IN IU/MG) OF NATIVE, DENATURED AND RENATURED PREPARATIONS OF ALDOLASE OF YOUNG AND OLD RABBITS

	Activity		
Preparation	*Native*	*Denatured*	*Renatured*
Young	8–9	0	8–9
Old 1	2.8	0	2.4
2	7	0	7
3	3.6	0	3.6

The conditions of denaturation and renaturation are described in "Materials and Methods."

1–3 are the different animal's preparation numbers.

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the present study as well as earlier studies by UV difference absorption and fluorescence spectra (Demchenko and Orlovska, 1980) demonstrate the potential usefulness of spectroscopic methods in gerontological research of protein structure at the molecular level.

The molecular basis of the differences of the helical content between the same protein isolated from young and old animals may have significant differences of helical-forming potentials of specific amino acids resultant from sequence changes or amino acid modifications before the native enzyme molecule is folded. Therefore, the logical development of this work is the study of the protein folding-unfolding *in vitro* (denaturation and renaturation).

The studies of aldolase denaturation and renaturation were also undertaken to test the hypothesis of Rothstein (1977) which suggests that the altered protein molecules observed in aging do not undergo any changes of covalent structure, but are simply conformational isomers. The theory of protein structure and of the pathways of protein synthesis and self-organization does not rule out the possibility of alternative folded structures, because the direction of the folding can be controlled both thermodynamically and kinetically (Greighton, 1978). The native protein conformation is at equilibrium and corresponds to absolute or relative minimum of free energy. But under specific conditions, some of these minima may be inaccessible kinetically or have a small possibility. As a result, there may exist conformational isomers, the reversible transition between which is limited by high energetic barriers. They are revealed in monomeric insect hemoglobins (La Mar, 1980) and differ by 180° turn of hemine group relative to γ-α-meso-axis. The transition between them is possible under extreme conditions (prolonged incubation at pH $>$ 10 and 40°C). Light chain immunoglobin G dimers can be obtained in two conformer forms (Edmundson *et al.*, 1978). In hemoglobin mutant Hb Cranston, the two components Cr_2 and Cr_1 with identical amino acid sequence are revealed, Cr_2 may slowly turn to Cr_1 (McDonald *et al.*, 1980). Three different conformations of heme and carbonyl group were shown for carboxyhemoglobin (Makinen *et al.*, 1979). The folding of native protein structure from the denatured forms *in vitro* may result in formation of conformers which are not identical to initial native enzyme. This is observed even in the case of a very small protein, pancreatic trypsin inhibitor (Kosen *et al.*, 1981). Altered substrate specificity was found for catalase (Marklund, 1973). The oxidation of reduced lysozyme in the air brings about the formation of anzymically active components that differ by incorrect arrangement of

disulfide bonds (Acharya and Taniuchi, 1976). In a senescent organism, the altered environment and specific regulatory conditions during the protein synthesis may result in altered protein conformation which could be retained during the protein isolation from the tissue. Another possibility, proposed by Rothstein (1977) is that the conformational isomers are formed during the prolonged "dwell time" of enzyme molecules in the tissues of old animals.

To test the hypothesis of Rothstein (1977) the unfolding-refolding experiments were performed with nematode *Turbatrix aceti* enolase which showed significant age-dependent differences in structure and activity (Sharma and Rothstein, 1978). After the denaturation in guanidine hydrochloride and renaturation the properties of old and young nematode enolase become similar and correspond to the properties of an old form (including the significantly decreased activity). These results do not exclude completely the possibility of primary structure modifications responsible for the differences observed, that is, the complete recovery in the case of "old" enzyme and incomplete recovery in the case of "young" one. In the present work on another object—the rabbit aldolase—we show that on refolding of unfolded protein the initial conformation is restored with the recovery of age-dependent differences. This result is in accord with a notion that definite covalent structure modifications, rather than isomerisation, are responsible for the observed age differences.

Assuming the gradual post-translational covalent structure modifications (Gershon *et al.*, 1979) or conformational isomerization during the prolonged "dwell time" (Rothstein, 1977) to be the origin of the effects in aging, one would expect the degree of modification to vary among individual enzyme molecules depending on their respective "dwell time" in the tissue. This would result in heterogenous populations of enzyme molecules ranging from completely intact to completely modified forms. To our knowledge, there are at least two sets of observations that exclude the possibility of the "old" enzyme to be a mixture of native and modified species. 1) In the studies of rabbit muscle aldolase conformational transitions at the increase of temperature (Demchenko and Orlovska, 1980), the sharp transition for the "old" enzyme was observed to be significantly shifted to higher temperatures and no transition occurred at the lower temperature where the denaturation of the "young" enzyme takes place. 2) The investigation of rat muscle glyceraldehide-3-phosphate dehydrogenase (Gafni, 1981) showed that in the presence of ATP that removes the negative cooperativity of NAD^+ binding, all four binding sites of the "old" enzyme display identical affinity towards NAD^+. These results could be better explained by the following hypothesis: in an aging organism the protein covalent structure post-synthetic modifications of synthetic amino acid substitutions take place prior to the folding of native conformation. This influences the folding pathway and results in a native protein with an altered conformation that may influence the different properties, including enzyme activity.

To illustrate the hypothesis the following scheme may be proposed:

Altered sequence changes or post-synthetic modifications → **Altered folding pathway** → **Altered conformation of a native enzyme**

The enzymes with an altered conformation may be isolated and studied *in vitro*. Being unfolded and refolded by different influences they follow the initial folding pathway and the conformation restored retains the conformational differences between "young" and "old" enzymes. In the present studies this phenomenon is observed on rabbit muscle aldolase.

The presented scheme is in accord with the experimental data of this work. Still, it may not be unique for interpreting the various experimental data of protein alterations in the aged organisms (Demchenko and Orlovska, 1981). In cells and tissues with extinct protein metabolism, such as red blood cells or eye lenses, the gradual accumulation of modified proteins may play the principal role.

Our data suggest the age-dependent alteration of protein structure to originate at the stage of protein synthesis or initial post-synthetic modifications. The existing information does not allow to draw definite conclusions on the nature of these alterations. In this respect, the study of the primary structure of altered proteins in aging is an essential present day problem.

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SUMMARY

The significantly increased helical content is observed in muscle aldolase molecule of old rabbits. The unfolding and refolding of protein conformation followed by circular dichroism, fluorescence and enzyme activity showed the recovery of initial conformation after the denaturation. The protein folds into the form that existed prior to denaturation—"young" into "young" and "old" into "old"—the conformational differences between them being restored. This suggests that the primary structure modifications prior to the folding of the native protein conformation are the origin of the age-dependent differences of aldolase structure and function.

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AGE-RELATED CHANGES IN SULFATION OF BASEMENT MEMBRANE GLYCOSAMINOGLYCANS

MARGO P. COHEN* and LING KU

University of Medicine and Dentistry of New Jersey, Department of Medicine, 100 Bergen Street, Newark, New Jersey 07103

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Abstract—Alterations in heparan sulfate, the principal glycosaminoglycan species in glomerular basement membrane, have been proposed as contributory to permeability changes in nephropathic disorders. In the present study, the extent of sulfation of glycosaminoglycans isolated from pronase digests of human glomerular basement membrane, obtained from subjects 15–89 years old, was examined. The sulfate to hexuronic acid ratio significantly diminished as a function of increasing age. These results suggest that decreased sulfation of glycosaminoglycans and consequent reduction in net negative charge alters permselective properties of the glomerular basement membrane with aging.

INTRODUCTION

THE IDENTIFICATION of glycosaminoglycans (GAG) in glomerular basement membrane (Kanwar and Farquhar, 1979a; Cohen *et al.*, 1981; Parthasarathy and Spiro, 1981) has prompted several studies concerning the role of this class of molecules in maintaining the integrity of the capillary filtration barrier and in the altered transglomerular permeability associated with certain nephropathic processes. Heparan sulfate is the predominant GAG species in basement membranes (Kanwar and Farquhar, 1979a; 1979b; Cohen and Surma, 1981), although small amounts of chondroitin sulfate and dermatan sulfate have also been found (Brown *et al.*, 1981). It is a polyanion possessing a net negative charge, which is believed to influence electrochemical properties and to contribute to the charge selective nature of the glomerular capillary barrier (Brenner and Hostetter, 1978; Kanwar and Farquhar, 1979c). Removal of heparan sulfate by an *in situ* enzymatic digestion leads to a dramatic increase in the permeability of glomerular basement membrane (GBM) to native ferritin (Kanwar *et al.*, 1980). Progressive neutralization of the negatively charged GAG sites in GBM by perfusion with high molarity buffers results in clogging of the membrane and impairs its ability to act as a selective filter (Kanwar and Rosenzweig, 1982). Thus, the decreased incorporation of [^{35}S]-sulfate into basement membrane GAG by glomeruli of steptozotocin-diabetic rats (Cohen and Surma, 1981), the diminution in hexuronic acid

*Address correspondence to: Margo P. Cohen, M.D. Professor and Director Division of Endocrinology and Metabolism UMDNJ-New Jersey Medical School, 100 Bergen Street, Newark, New Jersey 07103

content of human diabetic GBM (Parasarathy and Spiro, 1982), and the reduction in the amount of heparan sulfate-containing proteoglycan in the basement membrane of tumors transplanted to diabetic mice (Rohrbach *et al.*, 1982) are findings in recent studies which implicate changes in the GAG of GBM in the increased permeability of the glomerular filtration barrier associated with diabetes.

Several considerations prompted the present investigation, in which we examined the influence of age on GAG in GBM purified from human renal cortex. First, age-related pathologic and compositional changes in GBM similar to those reported in diabetes by some investigators have been described (Smalley, 1980; Langner *et al.*, 1981; de Bats *et al.*, 1982; Taylor and Price, 1982). Second, the effect of diabetes on certain cellular processes has been likened to premature aging. Third, changes in matrix molecules analogous to those associated with diabetes may accompany the glucose intolerance which occurs with aging. Finally, if renal function progressively declines with age (Brenner *et al.*, 1982), biochemical and/or structural correlates of certain physiologic alterations might be expected. We now report that the extent of sulfation of GAG in human GBM diminishes with age.

MATERIALS AND METHODS

Preparation of basement membranes

Human renal cortex was obtained under the auspices of the National Diabetes Research Interchange. Tissue was either perfused in preparation for potential transplant use, or was removed post-mortem within six hours of death and placed in a solution of Tris-buffered saline containing protease inhibitors, and frozen for shipment. After thawing, glomeruli were isolated by differential sieving, and the basement membranes purified by osmotic lysis and sequential detergent treatment as previously described (Carlson *et al.*, 1978; Cohen and Surma, 1980).

GAG isolation

The basement membranes were digested with pronase (1 mg/ml with an enzyme:protein ratio of ≈1:5) in 0.12M Tris HCl, pH 7.2, containing 0.01M $CaCl_2$ for 24 hours at 37°C (Linker and Hovingh, 1973). Digested proteins were removed by precipitation with 5% trichloroacetic acid (TCA), and the supernatant was extracted twice with several volumes of ether to remove TCA. The GAG in the aqueous solution were then precipitated with 1% cetylpyridinum chloride (CPC), washed, and reprecipitated with absolute ethanol.

Sample analysis

Following solubilization in water, aliquots of the GAG isolates were taken for the determination of uronic acid by the carbazole reaction (Bitter and Muir, 1962), and for measurement of sulfate by the rhodizinate method (Terho and Hartiala, 1971) after hydrolysis of the ester sulfates in 1N HCl. Glucuronic acid (5–50 ug) and K_2SO_4 (.01–.025 umol) were used as standards. For calculation of molar ratios, uronic acid was converted to GAG content based on a repeating disaccharide unit of Mr ≈ 460 (Parthasarathy and Spiro, 1982).

RESULTS

Pertinent patient data and results of individual analyses of the GBM samples for hexuronic acid and sulfate are presented in Table 1. Uronic acid content ranged from 0.7 to 6.6 ug/mg dry weight of GBM (0.0014 to 0.0142 umol GAG/mg dry weight). There was no significant correlation between age and uronic acid content. Sulfate content ranged from 0.23 to 2.1 ug/mg dry weight of GBM (0.0024 to 0.0217 umol/mg dry weight), and also did not show significant positive or negative correlation with age. Calculation of the molar sulfate to uronate ratios, however, revealed a significant diminution ($r = -0.7$) of this ratio with age (Figure 1), indicating that the extent of sulfation of GAG residues decreases with advancing age.

TABLE 1. SULFATE AND URONATE CONTENT OF GBM

Patient	*Age*	*Uronic Acid*	*Sulfate*	*SO_4/u.a.*
1	15	2.395	0.630	1.26
2	18	2.731	0.712	1.25
3	20	2.384	0.818	1.65
4	22	4.289	1.226	1.38
5	24	0.663	0.264	1.91
6	28	1.294	0.449	1.66
7	29	1.347	0.520	1.85
8	47	6.552	2.084	1.52
9	49	3.924	0.824	1.01
10	58	2.302	0.566	1.18
11	68	2.306	0.228	0.47
12	89	4.615	0.769	0.80
	Mean ± SEM	2.9 ± 0.48	0.76 ± .14	1.33 ± .12

Sulfate and uronate contents expressed as ug/mg dry weight of GBM. Age given in years. For calculation of molar ratios, uronic acid was converted to GAG based on a repeating disaccharide unit of $M_r \approx 460$ as discussed in the text.

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DISCUSSION

Glycosaminoglycans have only recently been identified as integral constitutents of glomerular basement membrane, and hence understanding of their role in, and response to, physiologic and pathologic processes is just emerging. Defined as anionic sites by their ability to bind cationic probes (Kanwar and Farquhar, 1979b; 1979c), GAG can complex with or trap different molecules and may modulate their passage by affecting basement membrane permeability (Brenner and Hostetter, 1978; Kanwar *et al.*, 1980). It is believed

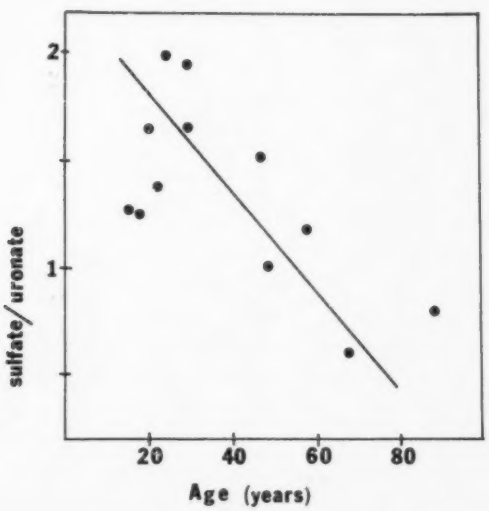

FIG. 1. Molar sulfate to uronate ratios, calculated from the data in Table 1, plotted as a function of age. $r = -0.71$

that GAG, by virtue of their net negative charge, influence electrochemical properties of the extra-cellular matrix (Comper and Laurent, 1978) and the charge-selective nature of the capillary filtration barrier (Kanwar and Farquhar, 1979a; 1979b; 1979c). Sulfate groups confer a negative charge to heparan sulfate, the predominant GAG in GBM (Parthasarathy and Spiro, 1981; Kanwar and Farquhar, 1979b; Cohen *et al.*, 1981; Kanwar *et al.*, 1980). A decrease in the number of sulfate groups linked to the disaccharide backbone would be expected to result in reduced electronegativity and, consequently, compromised integrity and charge-selectivity of the filtration barrier.

The sulfate and uronate contents of GAG isolated from unfractionated, detergent-treated human basement membrane observed in the present study are comparable to those reported in the literature for similarly prepared rat GBM (Kanwar and Farquhar, 1979b). Molar sulfate to uronate ratios were in the range of those reported for heparan sulfate isolated from dog, rat, and bovine GBM (Parthasarathy and Spiro, 1981; Linker *et al.*, 1981), in which sulfate to uronate ratios of 0.7, 1.1 and 0.9, respectively, have been found. The observation that sulfate to uronate ratios diminished with age indicates that the disaccharide unit of the GAG is undersulfated with advancing age. Reduced sulfate content of GAG in diabetic membranes has been ascribed to decreased incorporation (Cohen and Surma, 1981), accelerated removal (Ou *et al.*, 1979), and/or inhibition of N-deacetylase or N-sulfotransferase modifications of the N-acetylglucosamine residues (Kjellen *et al.*, 1983). Similar changes may be operative with advancing age and contribute to alterations in renal function in older populations.

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MECHANISM OF THE AGE-RELATED DECLINE IN LYMPHOCYTE PROLIFERATION: ROLE OF IL-2 PRODUCTION AND PROTEIN SYNTHESIS

H. TAK CHEUNG, JR-SHIN TWU, and A. RICHARDSON

Departments of Biological Sciences and Chemistry, Illinois State University, Normal, Illinois 61761

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Abstract—Although an age-related decline in mitogen-induced proliferation in spleen lymphocytes has been reported by numerous investigators, the molecular mechanism responsible is unknown. In this study, we compared the mitogen-induced proliferation, IL-2 production, and protein synthesis in spleen lymphocytes isolated from 4, 12, 20, and 30 month-old male Fischer F344 rats. IL-2 production by Con A-stimulated lymphocytes, as determined by the ability of the culture supernatants to support the growth of cultured T cells, declined over 72% between 4 and 30 months of age. This decline in IL-2 production paralleled a similar decrease in proliferation. Early protein synthesis by Con A-stimulated spleen lymphocytes was determined by measuring the incorporation of [^{3}H]-valine into acid insoluble material, and this dropped 74% between 4 and 30 months of age. There was a strong correlation between the age-related decline in the three parameters tested. Based on these results, we propose that the age-related decline in protein synthesis may be the molecular basis for the similar decrease in IL-2 production and mitogenesis.

INTRODUCTION

THE INCIDENCE of malignancy (Goodpasture, 1918; Teller *et al.*, 1964; Gross, 1965) and infectious diseases (Hijmans and Hollander, 1977) increases dramatically with senescence in higher animals. Present evidence indicates that the decline in the function of the immune system is directly responsible for these changes (Walford, 1974; Hijmans and Hollander, 1977). It has also been suggested that the decline in the immune system is partially responsible for the aging process (Walford, 1969; Burnet, 1970; Toh *et al.*, 1973; Walford, 1974). However, the mechanism responsible for the decline in the immune system and the subsequent increased susceptibility to conditions resulting in malignancy and infectious diseases is unclear. The effect of age on the immune response of rat is relatively unknown; therefore, it is unclear whether a similar age-related decline in the rat immune system occurs as is found in mice. Information regarding the effect of aging on the immune system of rats is obviously important in comparing the age-related change in the rat immune response with other animals, such as mice and humans, and also to ex-

Address reprints requests to: Dr. H. Tak Cheung, Department of Biological Sciences, Illinois State University Normal, Illinois 61761.

amine the relationship between the age-related changes in the immune system and other tissues, for example, the liver, brain, etc.

In a previous study, we demonstrated that decline in mitogen-induced, for example, phytohemagglutinin (PHA) and lipopolysaccharide (LPS), lymphocyte proliferation occurred with increasing age (Cheung, *et al.*, 1981). The decline was greater with PHA, a T cell mitogen, than LPS, a B cell mitogen (Cheung *et al.*, 1981). In this study, we have investigated the possible mechanism responsible for the age-related decline in lymphocyte proliferation to the T cell mitogens, PHA and concanavalin A (Con A).

Research during the past decade has demonstrated that lymphokines, biologically active substances secreted by stimulated lymphocytes, are essential in the immunological processes. Recently, Gillis and Smith (1977) identified a lymphokine, interleukin 2 (IL-2), by its ability to maintain T cell clones continuously in culture. It has subsequently been demonstrated that a subset of T cells are triggered to secrete IL-2 by mitogens (Smith *et al.*, 1979), while a different subset of T cells, rendered responsive by the mitogen, are driven to undergo cell-proliferation by the binding of the IL-2 to their receptors (Smith *et al.*, 1979). Therefore, IL-2 is a lymphokine that is extremely important in the activation of lymphocytes upon exposure to antigens and mitogens. However, the relationship between IL-2 production and proliferation by mitogen-stimulated lymphocytes during aging is unclear. In this study, we measured IL-2 production, proliferation, and protein synthesis by spleen lymphocytes throughout the entire life span of the rats to gain an insight into the mechanism of the age-related decline in mitogenesis. Our results suggest that decline in protein synthesis might be the molecular basis for the age-related decline in mitogenesis.

MATERIALS AND METHODS

Animals

Fischer 344 male rats, ranging from 4 to 30 months of age, were obtained from the animal colony maintained by Charles River Breeding Laboratories, Wilmington, Massachusetts for the National Institute on Aging. These rats were barrier reared and have a mean survival of 26 months (Coleman *et al.*, 1977). After arrival, the rats were maintained at our institution in laminar-flow environmental cages (Hazelton System, Cincinnati, Ohio) for at least two weeks before use in experiments. The rats were given water and a commercial lab chow (Purina) *ad libitum* and were monitored weekly for infectious diseases, malignancy, and abnormalities. The rats used in the experiments showed no loss of body weight, which can be an indication of pathological changes.

Isolation and culturing of lymphocytes

Rats were killed by inhalation of ether, and the spleens were removed aseptically. Single cell suspensions were obtained by gently rubbing the tissue against sterile stainless steel wire mesh (100) into 10 ml of Dulbecco's phosphate buffered saline (PBS) (GIBCO, Grand Island, New York). Contaminating erythrocytes were lysed by treating the cell pellet with 5 ml of tris-ammonium chloride lysis buffer (0.18 M NH_4Cl, 0.017 M Tris, pH 7.2) for 5 minutes at room temperature. After washing with 10 ml PBS, the lymphocytes were suspended in one of the following media, which contained penicillin (100 unit/ml) and streptomycin (100 μg/ml): EHAA medium (Peck and Bach, 1973) supplemented with 0.5% homologous rat serum to measure lymphocyte proliferation, valine deficient EHAA medium supplemented with 0.5% homologous rat serum to measure lymphocyte protein synthesis, and Click's medium (Altick Associates, Hudson, Wisconsin) supplemented with 10% heat inactivated (56°C for 30 minutes) fetal calf serum (GIBCO), 25 mM Hepes buffer, 16 mM $NaHCO_3$, and 300 μg/ml L-glutamine to measure IL-2 production. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Assay for cell proliferation

Lymphocyte proliferation was measured as described previously (Cheung *et al.*, 1981). Lymphocytes, 5×10^5 cells per 0.2 ml of EHAA medium, were incubated with 5 μg/ml of PHA (Sigma Biochemicals, St. Louis,

Missouri) in flat bottom wells of a microtiter plate (Costar, Cambridge, Massachusetts) for 48 hours, and 1 μCi of [^{3}H]-thymidine (6.7 Ci/mmole, New England Nuclear, Boston, Mass.) was added to each well. After incubation for 6 hours, the cells were collected onto glass-fiber filters (Whatman Inc., Clifton, New Jersey) using a micro-cell harvester, and the radioactivity incorporated into the DNA was determined using a liquid scintillation counter.

Assay for IL-2 activity

Il-2 activity was determined as described by Gillis *et al.* (1978) with slight modification. The culture supernatants from resting and Con A (Sigma Biochemicals) stimulated lymphocytes were tested for IL-2 activity by the ability of the supernatants to sustain the growth of IL-2 dependent rat cultured T cells (CTC) as measured by the promotion of DNA synthesis. CTC, which had been growing for at least 10 days in the presence of IL-2, were washed free of growth medium and resuspended in the supplemented Click's medium and added to the wells of a round bottom microtiter plate (Costar) at a concentration of 2×10^4 cells per 0.1 ml. To each well, 0.1 ml of various dilutions of the supernatants was added. After incubation for 24 hours, 1 μCi of [^{3}H]-thymidine (6.7 Ci/mmole) was added to each well, and the cell suspension was incubated an additional 4 hours. The cells were collected onto glass fiber filters and the incorporation of [^{3}H]-thymidine into DNA was measured using a liquid scintillation counter. Over 95% of the CTC cultured in the presence or absence of IL-2 were viable as determined by eosin dye exclusion. Only background radioactivity was associated with the CTC cultured in the absence of IL-2.

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Lymphocyte protein synthesis

One ml cultures of lymphocytes (10^7 cells) were incubated for 15 minutes and then 10 μCi of [^{3}H]-valine (57 Ci/mmole, ICN, Irvine, California) were added to the cultures together with 7.5 μg of Con A in the stimulated cultures. The lymphocytes were incubated for 3 hours with the [^{3}H]-valine unless otherwise stated. 50 μl of 20% trichloroacetic acid was added to the cultures, and the resulting suspensions were incubated at 90°C for 10 minutes. The acid-soluble material was collected on glass fiber filters, and the radioactivity in the acid-insoluble material was determined using a liquid scintillation counter.

Statistical analysis of data

The analysis of variance was used to determine if the lymphocyte mitogenic response changed significantly with age, and the Student's t test was used to determine when two values were significantly different.

RESULTS

Using lymphocytes from eight to ten month-old rats, we determined the conditions required for Con A-induced IL-2 production. Con A was the mitogen chosen to induce IL-2 production because Con A has been used to induce IL-2 production in a previous study with rat lymphocytes (Gillis *et al.*, 1978). Figure 1 shows the effect of various concentrations of Con A on IL-2 production and lymphocyte proliferation. IL-2 production was maximal at a Con A concentration of 7.5 μg/ml. This concentration of Con A also gave the greatest stimulation of cell-proliferation. A similar optimum Con A concentration has been reported by other investigators for lymphocytes obtained from humans and mice (Alverez *et al.*, 1979; Bonnard *et al.*, 1980; Gillis *et al.*, 1980). Figure 2 shows the time course for IL-2 production and lymphocyte proliferation after Con A stimulation. IL-2 activity was detectable within three hours after Con A addition and peaked at 24 hours. Lymphocyte proliferation was not detectable until 18 hours after Con A addition and was maximal at 48 hours. These results are very similar to those obtained by Alvarez *et al.* with human lymphocytes (Alverez *et al.*, 1979).

The production of IL-2 was determined after incubating lymphocytes from rats of various ages with 7.5 μg of Con A per ml for 24 hours. Table 1 shows the levels of IL-2 activity in culture supernatants obtained from Con A stimulated and non-stimulated lymphocytes from 4, 12, 20, and 30 month-old Fischer F344 rats. All Con A stimulated lym-

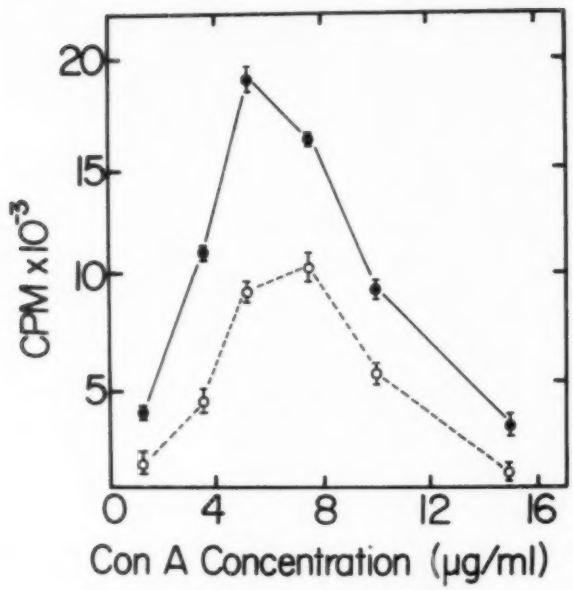

FIG. 1. The effect of various Con A concentrations on the proliferation (● ——— ●) and IL-2 production (○----○) by spleen lymphocytes. Proliferation was measured as the incorporation of [³H]-thymidine 48 hr after the initiation of culture. Culture supernatants were harvested 24 hr after initiation of culture, and IL-2 activity was measured by the ability of the supernatants to sustain IL-2 dependent CTC growth.

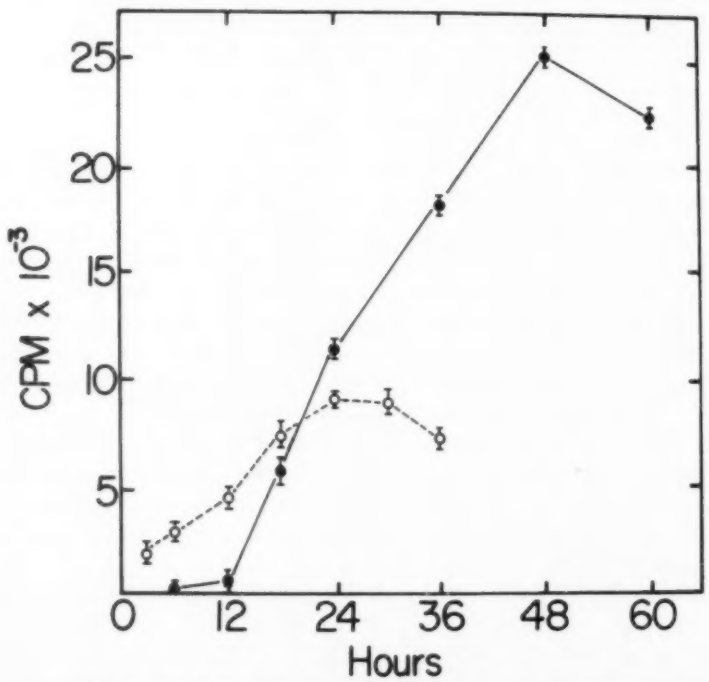

FIG. 2. Time course of Con A-induced (7.5 μg/ml) proliferation (● ——— ●) and IL-2 production (○----○) by spleen lymphocytes.

TABLE 1. IL-2 PRODUCTION BY CON A-INDUCED SPLEEN LYMPHOCYTES ISOLATED FROM MALE FISCHER 344 RATS OF VARIOUS AGES

		[³H]-Thymidine Incorporation by Cloned T Cells	
Age (Months)	*No. of Rats*	*Con A-stimulated (CPM ± S.E.)*	*Control (CPM ± S.E.)*
4	4	8,675 ± 206	268 ± 10
12	4	6,152 ± 190	178 ± 21
20	4	3,511 ± 106	201 ± 6
30	4	2,400 ± 68	222 ± 8

phocytes produced a significant amount of IL-2 activity, as determined by the ability of the culture supernatant to substain the growth of cloned rat T cells. The maximum IL-2 activity was found in the culture supernatant of lymphocytes from 4 month-old animals and a 72% decline in IL-2 activity was observed between 4 and 30 months of age. The decrease was observed primarily between 4 and 20 months of age, and only a slight decline, less than 10%, was found after 20 months of age. No IL-2 activity was evident in the culture supernatants from unstimulated lymphocytes for all ages tested.

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To determine if a correlation existed between the age-related decrease in IL-2 production and mitogen-induced lymphocyte proliferation, we measured lymphocyte proliferation by incubating lymphocytes with 5 μg of PHA per ml for 48 hours (Table 2). These conditions have been shown to be optimal for PHA-induced rat lymphocyte mitogenesis (Cheung *et al.*, 1981). PHA was chosen as the mitogen because in all the past research on the effect of age on lymphocyte mitogenesis PHA was the mitogen of choice (Pisciotta *et al.*, 1967; Mathies *et al.*, 1973; Foad *et al.*, 1974; Hung *et al.*, 1975a, b; Fernandez *et al.*, 1976; Cheung *et al.*, 1981). The PHA-induced mitogenesis of spleen lymphocytes, as measured by the incorporation of [³H]-thymidine, declined drastically with increasing age. The maximum mitogenic response was observed in lymphocytes from four month-old animals. The response declined in a linear fashion between 4 and 20 months of age, and at 20 months of age, the response was approximately 20% of the response at four months of age. However, only very slight decrease in mitogenesis was observed between 20 and 30 months of age. None of the unstimulated cultures showed any significant amount of incorporation above the background value. Con A was also used in a few experiments as a mitogen to study the effect of age on lymphocyte mitogenesis. The age-related decline in Con A-induced spleen lymphocyte proliferation was similar to that observed for PHA (data not shown).

TABLE 2. CELL PROLIFERATION BY PHA-INDUCED SPLEEN LYMPHOCYTES ISOLATED FROM MALE FISCHER F344 RATS OF VARIOUS AGES

Age (months)	*Stimulated Cultures (CPM ± S.E.)*	*Unstimulated Cultures (CPM ± S.E.)*
4	28,896 ± 1,356	672 ± 22
12	19,869 ± 876	693 ± 74
20	9,535 ± 376	782 ± 60
30	5,492 ± 1,070	651 ± 128

Each group consisted of 4 rats.

Because IL-2 is protein in nature, we measured the protein synthetic activity of Con A-stimulated and non-stimulated lymphocytes from rats of various ages in order to gain an insight into the molecular mechanism responsbile for the age-related decline in IL-2 production. Several investigators have shown that a burst in lymphocyte protein synthesis is one of the early events in mitogenic stimulation (Kay *et al.*, 1969; Ahern *et al.*, 1973; Wettenhall and London, 1974, 1976, 1977; Van Eijk and Muhlaradt, 1977). Figure 3 shows incorporation of valine into protein by lymphocytes immediately after Con A addition. [^{3}H]-valine incorporation by Con A-stimulated and nonstimulated lymphocytes was similar during the first hour of stimulation; however, after 3 hours of incubation, [^{3}H]-valine incorporation by Con A-stimulated lymphocytes was approximately threefold greater than for nonstimulated lymphocytes. Therefore, we also observed that a burst in protein synthesis occurred very shortly after mitogen stimulation. Such a biochemical change could be important in the synthesis of early proteins, for example, IL-2, that are important in proliferation. Table 3 shows the levels of early protein synthesis by spleen lymphocytes from animals of different ages. Protein synthesis by mitogen stimulated lymphocytes was highest in four month-old rats. A significant decrease in protein synthesis was observed between 4 and 12 months of age; however, the maximum decline occurred between 12 and 20 months of age. Only a slight change was observed after 20 months of age. The protein synthetic activity of the unstimulated lymphocytes was low but

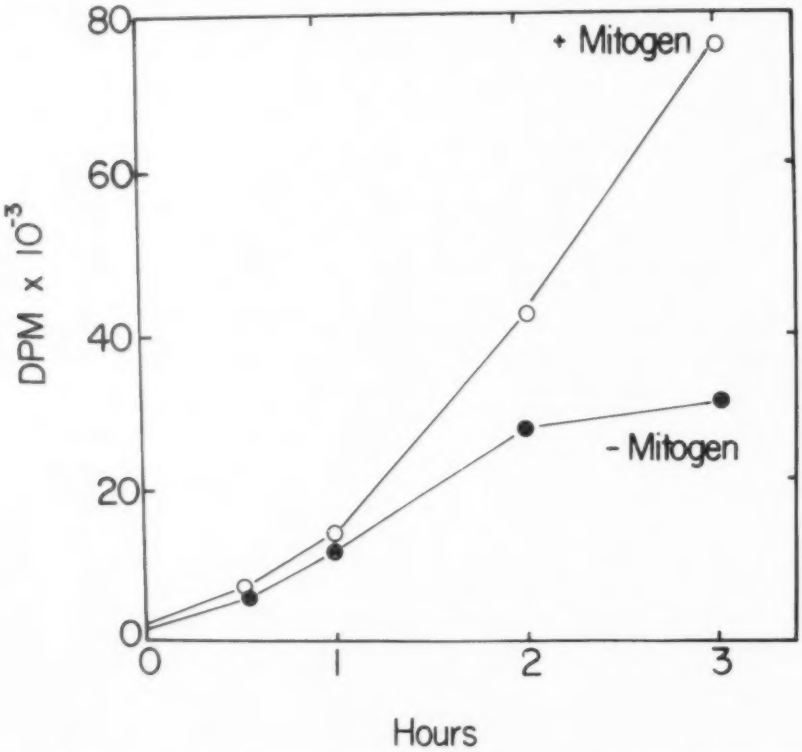

FIG. 3. Time course of [^{3}H]-valine incorporation in resting (● ——— ●) and Con A-induced (7.5 μg/ml) (○----○) spleen lymphocytes.

TABLE 3. PROTEIN SYNTHESIS BY CON A-INDUCED AND RESTING SPLEEN LYMPHOCYTES ISOLATED FROM MALE FISCHER F344 RATS OF VARIOUS AGES

		[^{3}H]-Valine Incorporations	
Age (Months)	*No. of Rats*	*Stimulated Cultures (DPM ± S.E.)*	*Unstimulated Cultures (DPM ± S.E.)*
4	4	27,567 ± 284	5,089 ± 82
12	4	22,789 ± 567	4,897 ± 294
20	4	10,899 ± 603	4,078 ± 446
30	4	7,083 ± 744	2,890 ± 306

significantly above background incorporation. It is interesting to note that protein synthesis by unstimulated lymphocytes remained relatively constant between 4 and 20 months of age; however, a significant decrease was observed between 20 and 30 months of age. This might represent a defect important in the cell's basic biochemical processes in addition to its inability to respond to environmental stimulation.

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To gain an insight into the molecular mechanism responsible for the age-related decline in mitogenesis, we compared the age-related changes in the three parameters studied (proliferation, IL-2 production, and protein synthesis). Figure 4 shows that the decrease in the

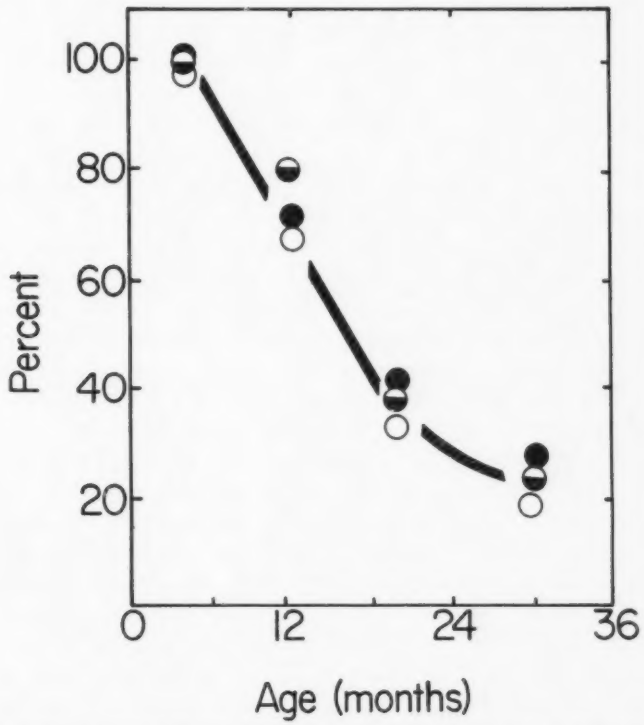

FIG. 4. The effect of donor age on cell proliferation (●), IL-2 production (○), and protein synthesis (◒) by spleen lymphocytes isolated from male Fischer F344 rats. The data were obtained from Table 1, 2, and 3.

three parameters as a function of age was similar. The magnitude of the decline between 20 and 4 months of age was 36% for proliferation, 43% for IL-2 production, and 41% for protein synthesis. Lymphocyte proliferation and IL-2 production were measured 48 and 24 hours after the addition of the mitogen. On the other hand, protein synthesis was measured three hours after mitogen addition. Because the age-related decrease in mitogen induced protein synthesis was an early defect in the process of mitogenesis, it could play a central role in the decline in the ability of lymphocytes to respond to mitogens. A decrease in the early synthesis of proteins required for mitogenesis, for example IL-2, would provide a molecular basis for how the immunological activity of lymphocytes declines with increasing age.

DISCUSSION

The age-related decline in lymphocyte mitogenesis has been reported by numerous investigators in humans (Pisciotta *et al.*, 1967; Foad *et al.*, 1974), mice (Hung *et al.*, 1975a, b; Mathies *et al.*, 1973), hamsters (Mathies *et al.*, 1973) and by our laboratory in rats (Cheung *et al.*, 1981). Both the T cell and B cell mitogenesis declines with age, but the decline in T cell response is greater than the decline in B cell response (Cheung *et al.*, 1981). The age-related decline of the T cell response to mitogens parallels the decline of the cell-mediated and antibody-mediated immunity *in vivo*. Because proliferation of the lymphocytes is necessary before their differentiation into effector cells, it is important to understand the underlying cause for such a decline. The recent findings on the importance of the lymphokine, IL-2, in the mitogen-induced proliferation of T cells led us to investigate the possible relationship between IL-2 production and the age-related decline in lymphocyte proliferation.

Using spleen lymphocytes from Fischer 344 rats, we found a close relationship between the age-related decline in the production of IL-2 by mitogen-activated lymphocytes and their proliferation. Both parameters decline in an almost linear fashion between 4 and 20 months of age and then decline to a lesser extent between 20 and 30 months of age. The striking similarity in the pattern of decline of these two parameters throughout the life span of the animals strongly suggests a causal relationship between them. Recently, Gillis *et al.* (1981) reported that lymphocytes obtained from elderly subjects produced less IL-2 and responded poorly to PHA compared to lymphocytes obtained from young subjects. Similar observations were also made in mice (Thoman and Weigle, 1981; Chang *et al.*, 1982) and in rats (Gilman *et al.*, 1982). The decline in IL-2 production by aged animals does not appear to be the result of suppression by suppressor cells (Thoman and Weigle, 1981; Chang *et al.*, 1982), increased absorption by responding cells (Chang *et al.*, 1982), or a decrease in helper cells (Chang *et al.*, 1982). Because Chang *et al.* (1982) recently showed that the addition of exogeneous IL-2 restored the mitogenesis of lymphocytes from the aged animals to that of young animals (Chang *et al.*, 1982), the decline in IL-2 production appears to be an important factor in the age-related decline in lymphocyte proliferation.

An age-related decline in protein synthesis appears to be a common phenomenon because it has been observed in a variety of organisms ranging from fungi to humans (Richardson, 1981a, b). However, no one has compared the protein synthetic activity of lymphocytes from young and old animals. This is ironic because several investigators have reported that protein synthesis is important in the initiation of mitogenesis: (a) a burst in

protein synthesis is one of the initial events observed after mitogen stimulation (Varesio *et al.*, 1980), and (b) inhibition of protein synthesis, particularly during the first few hours after mitogen stimulation, inhibits lymphocyte proliferation (Varesio and Holden, 1980) and other lymphocyte functions, for example, the production of lymphokines (Varesio *et al.*, 1980) and interferon (Vilcek and Ng, 1971) and the expression of insulin receptors (Helderman and Strom, 1979). An age-related decline in protein synthesis might play an important role in the molecular mechanism underlying the age-related decline in lymphocyte proliferation because a decline in protein synthesis would be expected to affect the ability of lymphocytes to produce IL-2 as well as other proteins that are important in the immune response, for example, IL-2 receptors and other lymphokines.

In our present study, the protein synthetic activity of unstimulated lymphocytes decreased only slightly (5%) with increasing age. However, a 74% decrease in protein synthesis was observed between 4 and 30 months of age after mitogen stimulation. The decline in protein synthesis paralleled the age-related decline in mitogen induced lymphocyte proliferation and IL-2 production. This is the first evidence linking a decline in cell function, for example, mitogenesis, to the age-related decline in protein synthesis and suggesting that the decline in the protein synthetic activity of mitogen-stimulated lymphocytes might be the molecular basis for the age-related decline in mitogenesis.

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AGE-RELATED CHANGES IN NON-ENZYMATIC GLYCOSYLATION OF HUMAN BASEMENT MEMBRANES

MARGO PANUSH COHEN and VAN YU-WU

University of Medicine and Denistry of New Jersey, Department of Medicine
100 Bergen Street, Newark, New Jersey 07103

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Abstract—Age and diabetes-related changes in the extent of non-enzymatic glycosylation of two anatomically distinct human basement membranes were examined. The amount of glucose in ketoamine linkage to glomerular basement membrane, measured by the thiobarbituric acid reaction, correlated positively and significantly with age. For analysis of lens capsule basement membrane, adsorption to agarose-linked phenylboronate was used. The ability of this resin to completely discriminate non-glycosylated from glycosylated residues was first documented by separating synthetic preparations of radioactive glucitol-lysine and glucitol-hydroxylysine, the glucosylamines formed with non-enzymatic glycosylation of collagen, from the respective free amino acids by affinity chromatography on and batch adsorption to phenylboronate. The extent of non-enzymatic glycosylation of lens capsule basement membranes correlated with age in both non-diabetic and diabetic samples, and the slopes of the lines for age versus glycosylation were similar in both groups. The calculated line for diabetic specimens, however, was displaced about 15 years to the left, compatible with premature aging. The results indicate that non-enzymatic glycosylation can be accurately assessed in minute amounts of tissue by phenylboronate adsorption, and provide evidence that human basement membrane is subject to increased glycosylation with aging and diabetes.

INTRODUCTION

NON-ENZYMATIC GLYCOSYLATION is a condensation reaction between carbohydrate and free amino groups at the N-terminus or ϵ-amino groups of lysine and hydroxylysine residues of proteins. The reaction is proportional to time, temperature, pH, and glucose concentration (Fluckiger and Winterhalter, 1976; Spicer *et al.*, 1979; Cohen *et al.*, 1981); the availability of free glucose and the half-life of individual proteins also influence the extent of glycosylation (Higgins *et al.*, 1982). Following the demonstration that hemoglobin undergoes post-ribosomal non-enzymatic glycosylation *in vivo* (Bunn *et al.*, 1979), numerous studies documented that diverse proteins are subject to this reaction under physiologic conditions, and that their degree of glycosylation is increased in the diabetic state with attendant hyperglycemia. The list of such proteins is growing and now includes albumin (Day *et al.*, 1979; Guthrow *et al.*, 1979), erythrocyte membrane proteins (Bailey *et al.*, 1976), lens crystallins (Monnier *et al.*, 1979; Stevens *et al.*, 1978),

Address Correspondence to: Margo P. Cohen, M.D. Professor and Director Div. of Endocrinology and Metabolism New Jersey Medical School 100 Bergen Street Newark, New Jersey 07103

glomerular proteins (Chang and Noble, 1979), aortic (Rosenberg *et al.*, 1979), skin and tendon collagens (Schnider and Kohn, 1980; 1981), peripheral nerve protein (Vlassar *et al.*, 1982), lipoproteins (Schleicher *et al.*, 1981) and basement membrane collagen (Cohen *et al.*, 1980; 1981).

Several considerations prompted the present investigation, in which we examined the relationship between the level of glycosylation and age in basement membranes isolated from two anatomic loci; the lens capsule and the renal glomerulus. First, increased glycosylation may accompany the glucose intolerance which occurs with aging. Second, since the effect of diabetes on certain cellular processes has been likened to premature aging, increased glycosylation of proteins with relatively slow turnover might occur with age even in the absence of known glucose intolerance. Third, age-related pathologic and compositional changes in glomerular basement membranes have been described (Smalley, 1980; Langner *et al.*, 1981; de Bats *et al.*, 1982; Taylor and Price, 1982) which are similar to those reported in diabetes by some investigators. Finally, glycosylation in aged and diabetic human lens capsule membrane, an extracellular matrix recently reported to share in the thickening and increased synthesis characteristic of other basement membranes in diabetes (Fisher, 1980; Laurent *et al.*, 1981), has not previously been studied. We now report that the extent of non-enzymatic glycosylation in human glomerular and lens capsule basement membrane increases with age, that the glycosylation of lens capsule basement membrane is increased in diabetes, and that the latter finding is compatible with premature aging of this tissue in diabetes. We also describe a method suitable for analysis of the extent of non-enzymatic glycosylation in minute amounts of tissue.

MATERIALS AND METHODS

Preparation of basement membranes

Human renal cortex and lenses were obtained under the auspices of the National Diabetes Research Interchange. Tissue was removed post-mortem and kept in ice cold sterile saline until transferred to the NDRI, where it was placed in a solution of 0.154M NaCl, 0.05M Tris HCl, pH 7.4 containing protease inhibitors (0.025M EDTA, 0.001M benzamidine hydrochloride, 0.001M phenylmethylsulfonylfluoride, 0.01M N-ethylmaleimide), and frozen for shipment. After thawing, glomeruli were isolated by differential sieving and the capsules were stripped from the lenses, and placed in cold distilled water containing the above protease inhibitors. The basement membranes were then isolated by osmotic lysis and sequential detergent treatment as previously described (Carlson *et al.*, 1978; Cohen and Surma, 1980).

Glomerular basement membrane analysis

Glycosylation of glomerular basement membrane was estimated by measuring the chromogen produced by the reaction of 5-hydroxymenthylfurfural with thiobarbituric acid (Fluckiger and Winterhalter, 1976). Pure 5-hydroxymethylfurfural (Sigma Chemical Company) was used as standard. About 10 mg (dry weight) of purified glomerular basement membrane was used for each analysis.

Glycosylation of lens capsule basement membrane

The small amount of material (less than 1 mg dry weight) obtained in each sample of lens capsule basement membrane prohibited accurate measurement of glycosylation by the thiobarbituric acid reaction. We therefore examined the ability of phenylboronate, an affinity resin that complexes with cis-diol groups of sugar (Ferrier, 1978; Brownlee *et al.*, 1980), to separate glycosylated from non-glycosylated amino acids and adapted this technique to batch analysis for measurement of total glycosylated residues in lens capsule basement membrane.

Synthetic standards of glucitol-lysine and glucitol-hydroxylysine were prepared by incubating solutions of glucose and respective amino acid in a 2:1 molar ratio at room temperature for 72 hours in 0.1M potassium phosphate buffer, pH 7.4, and then reducing with sodium borohydride (Bailey *et al.*, 1976). D-6-[^{14}C]-glucose (9mCi/mmole) was added to the hexose solution to give a specific activity of 10uCi/mmole in the reacting mix-

tures. Excess borohydride was removed by acidification to pH 5.0 with excess Dowex-H+, followed by washing the resin with water and then product elution with 0.5N NH_4OH (Schwartz and Gray, 1977). The eluates were concentrated by rotary evaporation, applied to a column of Biogel P-2 that had been standardized with [^{14}C]-glucose and tryosine, and eluted with 30% acetic acid. The effluent, monitored for radioactivity and ninhydrin positivity, showed one major peak emerging before free glucose and amino acid in each synthetic preparation. These respective peaks were pooled, lyophilized, and applied to the amino acid analyzer (Beckman Model 118BL packed with W-2 resin), equipped with a stream divider that allowed portions of the effluent to be fed into a fraction collector for subsequent radioactive monitoring.

Phenylboronate separation of non-glycosylated from glycosylated residues was assessed by both affinity chromatography and batch adsorption of acid hydrolyzed samples of the synthetic standards that had been filtered on Biogel P-2. With the former method, synthetic standards were applied to a 0.8 × 10 cm column of phenylboronate linked to agarose (PBA-60; Bio-Rad) equilibrated with 50 mM HEPES buffer, pH 8.5, containing 10 mM $MgCl_2$. After washing with 50 ml of the alkaline HEPES buffer, which removes non-glycosylated amino acids, the glycosylated products were eluted with 0.1M acetic acid. Effluent was monitored for ninhydrin positivity and for radioactivity in a liquid scintillation counter. The adsorbed peak, eluted with acetic acid, was evaporated *in vacuo* and applied to the amino acid analyzer. With the latter method, aliquots of the synthetic preparations were added to 5 ml of a suspension of PBA-60 in the HEPES buffer described above. The resin had been washed several times with this buffer before final suspension. Samples were agitated on a rotary mixer for 30 minutes and then centrifuged at 2500 rpm for 10 minutes. The supernatants were collected, the resin twice mixed with 5 ml of the HEPES buffer and centrifuged, with collection of the supernatants from these washes. The three HEPES supernatants were combined, lyophilized and reconstituted in 1 ml of water. The resin was then agitated for 30 minutes in 5 ml of 0.1M acetic acid, centrifuged, and the supernatant collected. The acetic acid wash was repeated twice, with pooling of the three acetic acid supernatants, lyophilization, and reconstitution in 1 ml of water.

Glycosylation of lens capsule basement membrane was determined by batch adsorption on PBA-60 as described above. The purified basement membranes were hydrolyzed for 24 hours in 6N HCl at 110°C, evaporated to dryness *in vacuo,* and reconstituted in HEPES buffer for separation of glycosylated from non-glycosylated residues by batch analysis.

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RESULTS

Non-enzymatic glycosylation of human glomerular basement membrane was measured by the thiobarbituric acid reaction in 16 samples obtained from patients ranging from 15 to 70 years of age. The extent of glycosylation in these samples, measured as liberation of hydroxymethylfurfuraldehyde (HMF) and expressed as nmoles HMF per mg dry weight of purified glomerular basement membrane, is shown in Figure 1. Glycosylation significantly increased with age, with a correlation co-efficient $r = 0.71$ between age and level of glycosylation.

The adequacy and sensitivity of the PBA adsorption method was documented before subjecting lens capsule basement membranes to this procedure. This was provided by analysis of the synthetic glycosylamines, alone or mixed with free amino acid, before and after PBA adsorption.

Application of the Biogel P-2 filtered synthetic standards to the amino acid analyzer revealed two major ninhydrin peaks but only one radioactive peak in each sample. With glucitol-hydroxylysine preparations, ninhydrin positivity eluted as a doublet at 39.2 and 42.3 minutes; the only other ninhydrin positive material in this preparation eluted as a biphasic peak at 90.3/94.2 minutes, coincident with pure hydroxylysine standard (Figure 2). All of the radioactivity in [^{14}C]-glucitol-hydroxylysine eluted between 39 and 43 minutes; maximum radioactivity was collected at 42 minutes, coincident with the ninhydrin peak. With glucitol-lysine preparations, radioactivity eluted at 50.8 minutes, coincident with a single sharp ninhydrin peak; the only other ninhydrin positive material in this preparation appeared at 110 minutes, with an elution time identical to pure lysine standard (not shown). Thus while all of the incorporated radioactivity from [^{14}C]-glucose was associated with the synthetic glucosylamines, these preparations contained contaminating free amino acid (Lysine or hydroxylysine) even after filtration on Biogel P-2.

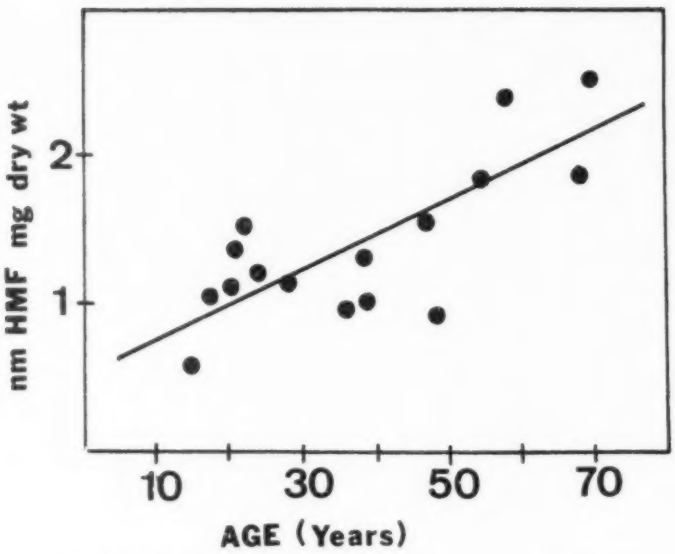

FIG. 1. Non-enzymatic glycosylation of human glomerular basement membrane. Analysis performed by measuring the chromogen produced by reaction of thiobarbituric acid with hydroxymethylfurfuraldehyde liberated upon heating under acid conditions of about 10 mg (dry weight) of each sample of purified GBM. Results expressed as nmoles HMF per mg dry weight of basement membrane. Correlation coefficient r = .071 between age and level of glycosylation.

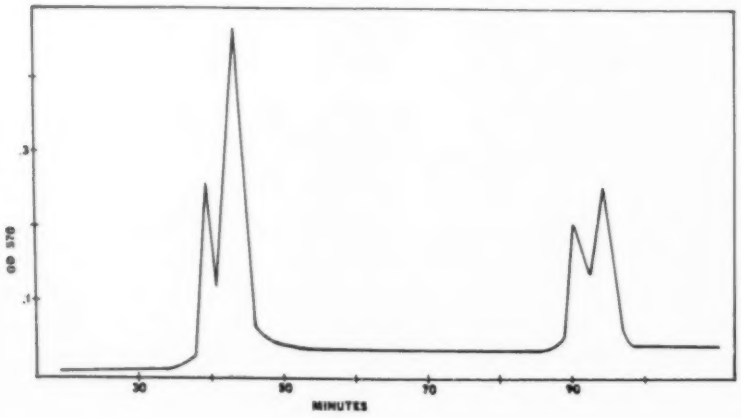

FIG. 2. Amino acid analysis of synthetic [^{14}C]-glucitol-hydroxylysine, prepared as described in the text and gel filtered on Biogel P-2. About 7,200 cpm were applied and 6,500 cpm were recovered in the analyzer effluent; all of the radioactivity eluted between 39 and 43 minutes, with a peak at 42 minutes. Elution was performed with 0.26N sodium citrate buffer, pH 5.25, until 92 minutes at which time the buffer was changed to 0.3N sodium citrate, pH 6.4, containing 0.6N sodium ion concentration, and run until 130 minutes. Initial column temperature was 45°C, and was changed to 55°C after 20 minutes.

Comparison of the integrated areas for lysine and hydroxylysine in these preparations with those for known amounts of the respective amino acid standards allowed calculation of the amount of free lysine in the glucitol-lysine preparation as 16% and of free hydroxylysine in the glucitol-hydroxylysine preparation as 7.5%.

Application of the Biogel P-2 filtered synthetic standards to the phenylboronate column resulted in separation of the ninhydrin positivity in each sample into two fractions: nonadsorbed and adsorbed. Under alkaline conditions, agarose-linked phenylboronate binds cis-diol groups of carbohydrate which can be subsequently eluted under acid conditions. Thus the non-adsorbed fraction represents free amino acid, whereas the adsorbed fraction represents glucosylamine (Figure 3). All of the radioactivity in synthetic glucitol-hydroxylysine (Figure 3) and glucitol-lysine (not shown) eluted in the adsorbed fraction. Subsequent application of this PBA-60 adsorbed fraction to the amino acid analyzer confirmed that all of the free hydroxylysine (Figure 4) or lysine (not shown) had been eliminated in the non-adsorbed material by the phenylboronate column. ~90% of the radioactivity in the PBA-adsorbed fraction was recovered after application to the amino acid analyzer, and all of the radioactivity coeluted with the respective glucosylamine ninhydrin peak.

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Separation of glycosylated from free amino acid by batch adsorption to PBA-60 was equally satisfactory, with all of the radioactivity appearing in the acetic acid eluates; about 90% of the radioactivity in glucitol-lysine or glucitol-hydroxylysine was recovered in each analysis. Measurement of ninhydrin positivity, using lysine or hydroxylysine as standard, revealed that 10% of the total ninhydrin positive material appeared in the HEPES eluates in each sample. The acetic acid eluates contained only one peak on amino acid

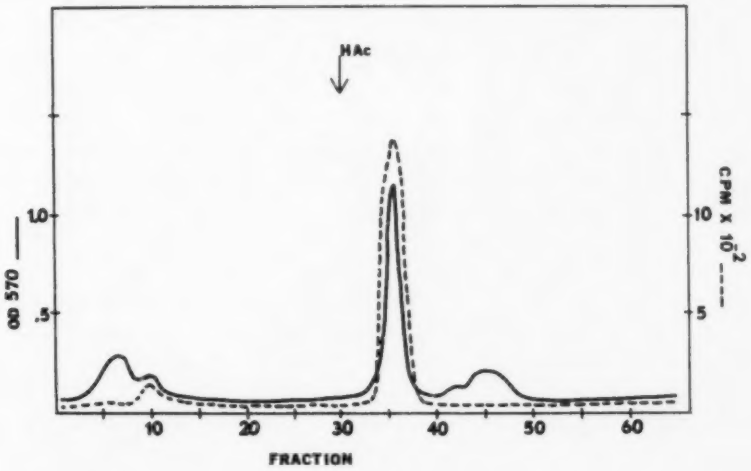

FIG. 3. Elution profile of synthetic [^{14}C]-glucitol-hydroxyiysine on agarose-linked phenylboronate. The column (0.8 × 10 cm) was equilibrated and washed with 0.05M HEPES, pH 8.5, to elute nonadsorbed material, and then eluted with 0.1M acetic acid (indicated by arrow) to recover adsorbed material. Fractions of 2 ml were collected, of which 0.2 ml was used for the ninhydrin reaction and 0.2 ml for measurement of radioactivity. About 88% of the applied radioactivity (21, 00 cpm) was recovered, virtually all of which eluted with the adsorbed fraction.

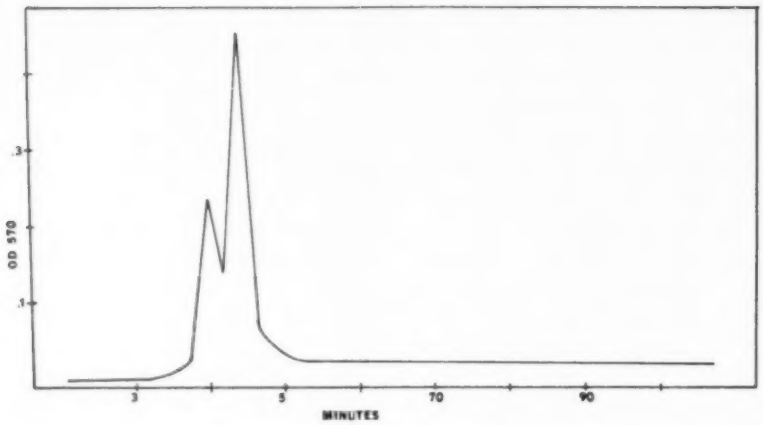

FIG. 4. Application of the adsorbed peak recovered from the PBA-60 column shown in Fig. 3 to the amino acid analyzer. Elution conditions as described in the legend to Fig. 2. All free hydroxylysine has been eliminated. About 90% of the applied radioactivity was recovered in the analyzer effluent; all of the radioactivity coeluted with the glucosylamine ninhydrin peak.

analysis, coincident with the elution times of glucitol-lysine and glucitol-hydroxylysine, respectively.

Acid hydrolyzed samples of lens capsule basement membrane (LCBM) from 11 non-diabetic and the 6 diabetic subjects listed in Table 1 were analyzed for glycosylated amino acids. Based on lysine standard in the ninhydrin reaction, glycosylated residues (glucitol-lysine plus glucitol-hydroxylysine in the acetic acid fraction) represented 1.6 to 5.2 percent of the total ninhydrin positive material (HEPES plus acetic acid fractions) in nondiabetic samples and 2.0 to 4.3 percent in diabetic samples. The ratio of glycosylated to non-glycoslated amino acids directly correlated with the age of the subject in both non-diabetic ($r = .73$) and diabetic ($r = .71$) samples (Figure 5). The slopes of the lines for age versus glycosylation ratio were similar for non-diabetic and diabetic samples, but the calculated line for diabetic specimens was displaced about 15 years to the left, indicating that the percent glycosylation in samples from diabetic subjects was greater than that in non-diabetic

TABLE 1. DIABETIC PATIENTS

Age	*Sex*	*Duration of Diabetes*	*Cause of Death*	*Diabetic Treatment*	*FBG (range)*[a]
60	M	7 years	Arrythmia	Diet	120–180
71	M	Unknown	Cardiac Arrest	Insulin	Unknown
75	M	5 years	Pulmonary Embolism	Insulin	Unknown
76	F	17 months	Unknown	Insulin	120–260
79	M	8 years	Anoxia	Insulin	180–220
89	F	15 years	Pneumonia; uremia	Oral agent	180–220

[a]FBG = fasting blood glucose; range given in mg/dl

Clinical information concerning duration of diabetes, FBG, treatment, etc. obtained from NDRI resources, including contacting family physician when possible.

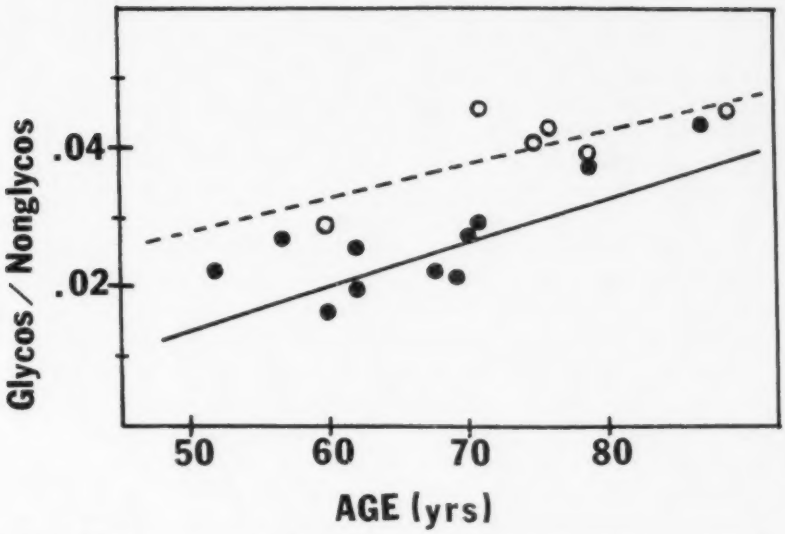

FIG. 5. Glycosylation of human lens capsule basement membrane, measured by adsorption to affinity resin. Results expressed as the ratio of μmoles of glycosylated to non-glycosylated residues calculated from standard curves using pure lysine as reference. Closed circles represent non-diabetic samples, open circles represent diabetic samples. For non-diabetic samples, r = .73 between age and level of glycosylation; for diabetic samples, r = .71.

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subjects of similar age (Figure 5). The mean values for glycosylation ratios in non-diabetic versus diabetic samples were significantly different (mean ± SEM = .0251 ± .0025 nondiabetic; .0398 ± .0026 diabetic). The ratio of glycosylated to nonglycosylated residues in the LCBM of an additional patient, age 22 at time of death from alcohol encephalopathy and with poorly controlled insulin dependent diabetes, was 0.033. This result, which was not included in the above calculations, was clearly above expected values extrapolated from the lines in Figure 5.

DISCUSSION

Synthetic [^{14}C]-labeled glucitol-lysine and glucitol-hydroxylysine can be separated from non-glycosylated amino acid residues by adsorption to agarose-linked phenylboronate. Amino acid analysis of the adsorbed peak, obtained after application to the PBA column of a mixture of the glucosylamine with unmodified lysine or hydroxylysine, demonstrated that this separation is specific and complete. Application of the method for batch analysis proved reliable and reproducible, with recovery of about 90% of the radiolabeled synthetic product in the acetic acid eluate and complete separation of the [^{14}C]-glucosylamine from free amino acid. The latter technique allowed simultaneous measurement in multiple samples of the amount of glycosylated amino acids in acid hydrolysates of insoluble proteins in minute amounts of tissue.

Studies with various proteins have indicated that glucose reacts with free amino groups at the N-terminus and ε-amino groups of lysine residues (Fluckiger and Winterhalter, 1976; Bunn *et al.*, 1975; 1979; Monnier *et al.*, 1979). Hydroxylysine, which derives from

peptide-bound lysine, retains a free ε-amino group and hence is also subject to non-enzymatic glycosylation (Cohen and Wu, 1981; Wu and Cohen, 1982). With few exceptions, this amino acid is unique to collagen and is particularly abundant in basement membrane collagens. Thus, glucose in ketoamine linkage with lysine, hydroxylysine and N-terminal residues of basement membrane proteins will all bind to phenylboronate and be measured in the adsorbed fraction. The finding that the total amount of glycosylated amino acid in lens capsule basement membrane measured with this method increases with age and diabetes agrees with results reported with other tissues using different methodologies (Stevens *et al.*, 1978; Monnier *et al.*, 1979; Rosenberg *et al.*, 1979; Schnider and Kohn, 1980; 1981; Cohen *et al.*, 1980; Dolhofer and Wieland, 1979; Chiou *et al.*, 1981).

The results reported herein demonstrate that glycosylation of two anatomically distinct basement membranes increases with age, and that human lens capsule basement membrane is subject to increased glycosylation in diabetes. They also describe methods for measurement of non-enzymatic glycosylation that are rapid, accurate, completely separate glycosylated from non-glycosylated residues in acid hydrolysates of insoluble proteins, and are suitable for use with minute amounts of tissue. These techniques should prove useful for assessing the impact of changes in blood glucose control on the level of non-enzymatic glycosylation in various tissue proteins with different turnover times and biologic half-lives.

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AGE AND OXYGEN TOXICITY RELATED FLUORESCENCE IN THE HONEY BEE THORAX

ROGER G. YOUNG and GENE ROBINSON

Department of Entomology, Cornell University, Ithaca, NY 14853

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Abstract—The blue fluorescent material in thoraxes of honey bees of known adult age from eclosion to 28 days was measured. The amount was greater in older insects and exposure to ozone (ppm) augmented the rate of accumulation. The nearly constant daylight flying by the bees and the ozone effect suggest radical peroxidative origin of the fluorescence.

The material was partially purified by chromatography. It is a relatively non-polar lipid.

INTRODUCTION

INSECTS THAT fly a lot have high metabolic rates and they soon lose the capacity to fly. This manifestation of aging is an example of the rate of living theory of aging (Sohal, 1981).

Some of the loss of function associated with aging may be the result of damage to lipids, which occurs by free radical attack on polyunsaturated fatty acids of membranes. Molecular oxygen is a radical that by chain reactions may form lipid peroxides that undergo scission to yield malonaldehyde and small hydrocarbons (Chio 1969; Dillard *et al.*, 1977). Schiff bases that can be formed between malonaldehyde and amino compounds are fluorescent in the range 430–470nm (blue). Bridges and Sohal (1980) have shown that in the housefly *Musca domestica*, at least part of the fluorescence depends on the presence of linoleic acid. Whether this age-related material should be termed lipofuscin is in question (Eldred *et al.*, 1982).

The worker honey bee *Apis mellifera* is an attractive organism for the study of this part of aging. Large numbers of individuals of uniform size, age and genetic makeup are available. Worker bees three or more weeks old spend most of the daylight hours in flight, which leads within six weeks to aged individuals whose flight muscles have been exposed to large quantities of oxygen. This results in fluorescence, membrane damage and ultimate decrease of function of the sarcosomes and other cell components (Young and Tappel, 1978).

The objective of this work was to study molecular oxygen-induced lipid damage by measuring the production of fluorescent material in ozone-treated and untreated worker honey bee flight muscle of known age, and to attempt the characterization of the age-related material.

MATERIALS AND METHODS

Bees were obtained from the colonies maintained at Dyce Laboratory for Honey Bee Studies at Cornell University during the summers of 1980 and 1981. They were hybrids of the races commonly used in commercial apiculture. In each replicate, 1200 bees were marked on the thorax with model enamel within 24 hours of emergence from combs that had been held in an incubator at 34°C for 24 hours prior to eclosion. Marking bees in this manner is a widely accepted and harmless procedure (Ribbands, 1952); furthermore a dead bee trap (Gary, 1960) did not yield a greater proportion of marked individuals during the study period. The marked bees were added to a colony of about 15,000 individuals. They were recaptured from combs at intervals for analysis of fluorescent material. If not used at once they were stored frozen. Only the thorax tissue, consisting of muscle cells, trachea and cuticle, was examined. For large-scale preparations bees were mixed with powdered dry ice and shaken in round cardboard ice cream cartons to fragment the bodies. The thoraces were then hand collected. It was also possible to freeze dry the whole insects before shaking (without dry ice) and collecting the separated thoraces.

Reagent grade solvents were distilled from glass and tested for fluorescence. Diethyl ether was dried over $CaCl_2$ then distilled from sodium metal. Other reagents were the best grade obtainable.

Extracts of fluorescent pigment for comparisons were made by grinding two bee thoraces three times with a total volume of 3.5 ml of a 3:2 v/v mixture of hexane and isopropanol, decanting each portion through glass wool. The combined extracts were washed with ⅓ volume of a solution of anhydrous sodium sulfate (Hara and Radin, 1978). The hexane layer was then dried over anhydrous sodium sulfate. Some extractions were similarly made with a 2:1 v/v mixture of chloroform methanol.

The maximum excitation and emission of the extracts were found to be at 360 and 425nm respectively when measured with an Aminco Bowman SP 125 spectrofluorometer. Slit widths were two and one millimeter respectively. The instrument was calibrated daily with a solution of 0.1 μg/m quinine in 0.1N H_2SO_4.

Fluorescent materials were separated by column chromatography (10 × 180 mm column) with silica gel (Bio-Sil 200–400 mesh, Bio-Rad Laboratories, Richmond, CA) that had been activated at 110° for an hour. Eluting solvents were collected automatically in 1.6 ml fractions and fluorescent materials were measured at the wavelength maxima. Esters and thiobarbituric acid reactive material were analyzed by the methods of Rapport and Alonzo (1935) and Young (1982) respectively.

Methyl esters of fatty acids were catalytically prepared and analyzed on a two meter column of 10% butanediol succinate at 180° and with flame ionization detection.

Silica gel plates were used for thin layer chromatography. They were washed with a mixture of chloroform methanol acetic acid 75:25:1, dried and activated for 30 mintues at 110° before use. Solvent system was diethylether, hexane, acetic acid 50:50:2. The reagent of Dittmer and Lester (1964) was used for phospholipid detection. Cholesterol and congeners were detected with Lieberman-Burchard reagent and 1:1 aqueous H_2SO_4. Fluorescent material was detected with a mercury vapor lamp with a Wood filter.

Ozone was generated by passing air over a 20 watt ultraviolet generating lamp enclosed in a 3-inch copper tube. The bees were held in wire mesh cages within a larger sealed polyethylene-lined container while the ozone-bearing air passed through. The ozone content was measured by the Saltzman (1964) method.

RESULTS AND DISCUSSION

Ozone effects

As seen in Figure 1 fluorescence at 360/425nm increased for three weeks in both the ozone-treated and control groups of age-marked bees, with higher levels of fluorescent material evident in the ozone-treated workers. The diminution of accumulation after three weeks may indicate a repair phenomenon, or more likely a selection process, that is bees with higher levels of oxygen-induced cellular damage due to either greater flight activity or ozone treatment may not have survived.

Newly emerged bees had relatively equal amounts of fluorescent material, presumably due to their genetic homogeneity. The increased variance in the amounts of fluorescence found in the older cohorts may have been the result of differences in worker bee activity. Although most bees spend their first three weeks working in the nest (so-called nurse bees), some begin foraging earlier (Ribbands, 1952). In our experiments some marked bees were observed foraging when they were 7 to 14 days old, and would therefore be expected to produce more fluorescent material than similar aged nurse bee sisters, but were not distinguishable when collected.

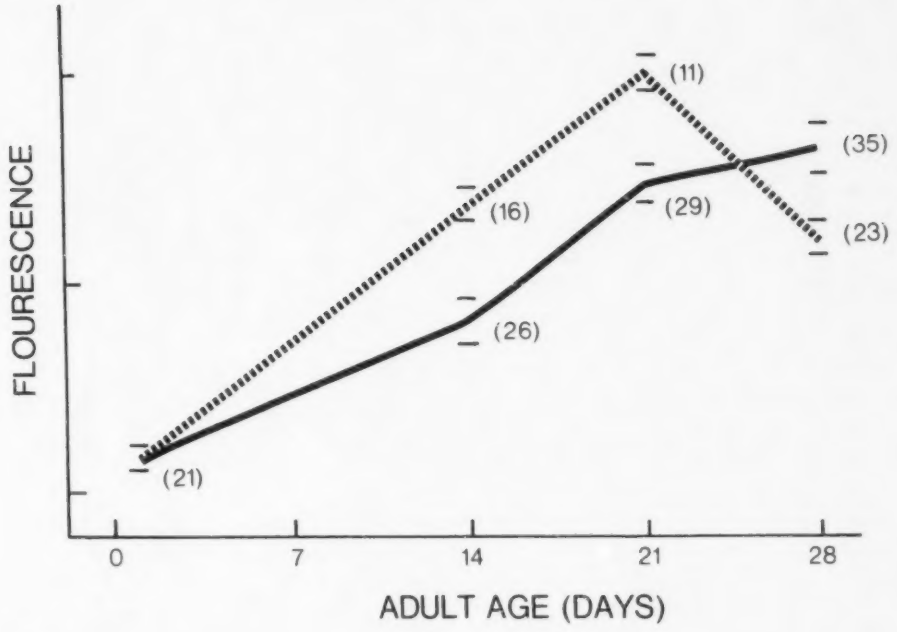

FIG. 1.
Ozone treated ------
Untreated —
Ozone treatment ~10 ppm for 20'
mean, plus and minus standard deviation ⊏
( ) - number of pairs of bees analyzed.
The data from 1980 and 1981 were combined.

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Experiments on fluorescent material

The use of thorax tissue that consists mainly of specialized mitochondria allows the study of relatively pure material, largely devoid of yellow pigments and of retinal. There are several blue fluorescent materials in bee thorax extracts, which can be separated by silica gel columns as seen in Figure 2 which illustrates only part of the elution scheme. Both more and less polar solvents elute esters and fluorescent material not shown in the figure.

Investigation of the properties of the age-related materials was focused on the sharply defined fluorescent and thiobarbituric acid reacting fraction which was eluted by 5% methanol in chloroform. It comprised less than 10% of the 360/425 fluorescence. When this eluate was chromatographed on silica gel TLC two blue fluorescent spots were apparent. The one that did not migrate ($R_f = 0$) with hexane ether was phospholipid and accounts for the ester content of that column fraction. When eluted from plates, then methylated, the fluorescence was lost as was the phospholipid staining property when it was rechromatographed. The saturated fatty acids of this phospholipid fraction had 10 to 16 carbon atoms.

The second fluorescent spot showed at $R_f = 0.6$. It was not altered by the methylation procedure. It did not react with Dragendorf's reagent or ninhydrin. It was stable to acid.

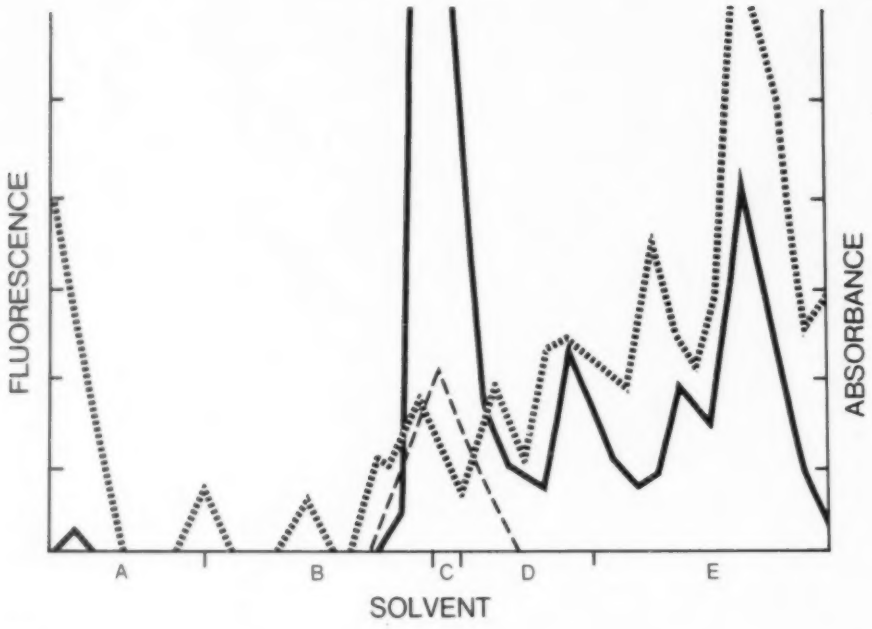

FIG. 2.
Fluorescence 360/425 —
Esters ------
Thiobarbituric acid reaction ----
Solvent A – 1% methanol in chloroform
B – 5% methanol in chloroform
C – 10% methanol in chloroform
D – 50% methanol in chloroform
E – methanol.

When eluted from the TLC plates it deposited on evaporation highly fluorescent small particles whose fluorescence was stable to heating in vacuum to 120°. It did not sublime. No useful information was obtained from mass spectroscopy. Recent use of high performance thin layer chromatography plates with staining by 50% H_2SO_4 revealed the presence of non-fluorescent impurities.

Hexane isopropanol extracts of lysosomes prepared from rat liver and from the gut of the larva of the Southern armyworm, *Prodenia eridania* (Young, 1982) and housefly thorax tissue all contain a component with the same fluorescence spectrum and thin layer chromatography behavior as the bee extracts described here. The amounts of fluorescence in young fly and bee thoraces are comparable on a fresh weight basis and the values in both insects were doubled two to three weeks after emergence. These findings suggest that the lipid portion of subcellular membranes is a common locus of the fluorescent material and that its quantity is age-dependent. Although the peroxidation of linear polyunsaturated fatty acids is accepted as a factor in aging, oxidation of cholesterol and its esters has apparently not been seriously considered. Smith (1982) has emphasized the ease of oxidation of cholesterol and indicated the number of products that have been found in tissues that can have resulted from oxidative and/or peroxidative degradation of cholesterol. A suggestion of this was found in spectrofluorimetric analysis of peroxidized cholesterol.

SUMMARY

The blue fluorescence in thorax tissue of honeybee has been shown to accumulate with adult age, apparently due to oxygen damage resulting from nearly constant flight. Exposure to ozone enhances the rate of production of the fluorescent material.

Some purification of the fluorescent lipid was achieved by column and thin layer chromatography. The migration and staining properties indicate that is is not phospholipid.

Acknowledgement—We thank Anita Meiklejohn for her assistance.

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HUMAN COLLAGEN DIGESTION: I. NATURE OF THE DIGESTION KINETICS AS A FUNCTION OF AGE AND STRUCTURE

JAMES S. HOLTON*, MORTON H. LITT, ROBERT R. KOHN, and CLIVE R. HAMLIN†

Department of Macromolecular Science (J.S.H. and M.H.L.), and Institute of Pathology, School of Medicine (R.R.K. and C.R.H.), Case Western Reserve University, Cleveland, OH 44106, U.S.A.

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Abstract—The digestion of human collagen by bacterial collagenase was studied in order to relate collagen structure to the digestion kinetics. The digestion was shown to be first order in collagen and in collagenase on given material from run to run. There was no product inhibition and no enzyme deactivation. However, the rate changed with age, and also within a run as a function of conversion. It was shown that the normalized time (time × enzyme concentration), to reach a given amount of digestion can be plotted as a linear function of the sample age, from 20 to 70 years.

The digestion broke down into two regions. The first was a rapid first order region which amounted to about 10% of the collagen. The second region could be graphed as a second order plot to obtain a straight line. This implied that the rate of digestion was functionally second order in collagen; first order in concentration and first order in conversion of collagen.

INTRODUCTION

THE MAJOR form of diaphragm tendon or dura mater collagen, type I, consists of two identical $\alpha 1$ chains and one $\alpha 2$ chain. Each contains approximately 1050 amino acids, 96% of which form sequences of the type -glycine-X-Y-. Approximately 30% of the X and Y amino acids are hydroxyproline, proline and alanine.

Insoluble collagen is formed by the introduction of intermolecular covalent crosslinks between monomeric collagen helices. At least a portion of these crosslinks involve lysine or hydroxylysine derivatives (Tanzer, 1973). Of the approximately 30 lysine or hydroxylysine residues per α chain, there are eight in registry which, theoretically, could generate intermolecular crosslinks (Fietzek and Kuhn, 1976). The total number of crosslinks have been postulated to increase in post-mature collagen with age (Kohn, 1978).

The properties and mode of action of Clostridium histolyticum collagenase (Clostridiopeptidase A, EC 3.4.4.19) on collagen has been reviewed (Mandl, 1961; Nordwig, 1971).

There are at least two major components of the enzyme. One is more active towards insoluble collagen and the other is more active toward soluble collagen. The sum of the ac-

*Present Address: ARCO Chemicals Company, 3801 West Chester Pike, Newton Square, PA 19073, U.S.A.
†To whom reprint requests should be addressed.

TABLE 1. POSSIBLE SITES OF CLEAVAGE BY COLLAGENASE

-Gly - Pro - (Hyp)	--- Gly - Pro - (Hyp)-[a]
-Gly - Pro - Y	--- Gly - Pro - (Hyp)-[a,b]
-Gly - Pro - Y	--- Gly - X - (Hyp)-[a,b]
-Gly - X - Hyp	--- Gly - Pro - Y -[c]
-Gly - X - Y	--- Gly - Pro - Y -[c]
-Gly - X - Y	--- Gly - Z - (Hyp)-[a,d]

[a]bracketed amino acids can be either proline or hydroxyproline
[b]X and Y cannot be glutamic acid
[c]Y cannot be glutamic acid
[d]Y and Z cannot be glutamic acid

tivities of the separate components is always less than the activity of the unfractionated enzyme or a pool of the components (Kono, 1968).

There are six possible sites of cleavage by collagenase (Table 1). Review of the α1 (I) sequence of collagen identifies 177 potential cleavage sites with a potential average product size of 5.7 amino acid residues (Hulmes *et al.*, 1973). This is similar to experimentally derived values. The potential product distribution from the review shows that over 95% of the products would be 15 amino acid residues in length or less.

Previous investigation of collagenase digestion of soluble collagen indicated that digestion results from two simultaneous first order reactions, one about four times faster than the other but accounting for only 10 to 20% of the digestion (Mandl *et al.*, 1958). Digestions of denatured collagen occurred only by the fast reaction (Chu and Lukton, 1974). Digestion of lightly crosslinked collagen gels (0.14% formaldehyde; 6 moles per mole of collagen) by bacterial collagenase has been found essentially identical to that of an uncrosslinked control. The resistance to digestion increased as the level of crosslinking increased, until 1.4% formaldehyde ($\sim$60 moles per mole of collagen) was reached, after which no digestion occurred (Harris and Farrell, 1972). Digestion of insoluble collagen by bacterial collagenase as a function of *in vivo* age has been investigated (Hamlin and Kohn, 1971; Hamlin *et al.*, 1978). The digestion was found to follow a simple first order mechanism only during the initial stages of the reaction. Two processes were proposed to occur: An initial rapid digestion, which predominates in young samples, and a slower digestion mechanism which accounts for an increasing fraction of the collagen digestion after maturation.

The present study, involving a more complete determination of the general digestion kinetic mechanism consistent with the properties of collagen and collagenase mode of action, was undertaken to provide a better understanding of the age-related changes in collagen.

MATERIALS AND METHODS

Purified 36.3-year-old human dura mater collagen was used for the detailed study of the enzymatic digestion parameters. Digestion data (volume of base versus time) for purified collagen samples of other ages, from dura and diaphragm tendon, were supplied by Hamlin and Kohn. The collagen purification procedure has been reported previously (Hamlin and Kohn, 1971; Hamlin *et al.*, 1975).

The purified collagen was assayed for moisture, ash, hydroxproline content and ninhydrin value. Greater than 95% of all protein found in the dura mater and diaphragm tendon samples was collagen. Two to four milligrams of carbohydrates were present per gram of protein.

Purified collagenase (Worthington Biochemical Corporation, CLSPA) was used without further purification. Activity values were checked. A sufficient quantity of enzyme was dissolved in distilled water, measured into ap-

propriate aliquots (0.250 to 2.000 ml) and stored at −70°C until used. All aliquots of a given enzyme lot were used within nine months of preparation with no apparent loss of activity during this time span.

The pH-Stat used was a Radiometer-A/S (Copenhagen NV, Denmark) system consisting of a PHM26 pH Meter, ATTT II Titrator, an ABU 13 autoburette (2.5 ml capacity), a TTA3 titration assembly with a Thermostat Jacket and vessels, and a SRB recorder. The digestions were done following a previously described procedure (Hamlin and Kohn, 1971; Zwolinski *et al.*, 1976).

Lyophilized collagen was suspended in 0.05M NaCl −0.05M $CaCl_2$ solution using a glass tissue grinder, or was sonicated for 15 seconds using an Artek Sonic Dismembrater. The suspension was equilibrated for a minimum of 15 minutes in the titration assembly to allow for temperature equilibration prior to addition of enzyme.

The acid formed by digestion of the collagen by collagenase was automatically titrated with 0.01M NaOH to maintain a constant pH in the reaction vessel. The vessel and digestion mixture were maintained at 37°C, a pH of 7.80 and under a water saturated nitrogen purge. Digestions were allowed to proceed from 15 minutes to 10 hours, depending on the experiment being done. Most were followed for 70 minutes.

A product inhibition test was performed by taking one digestion to completion. The reaction mixture was then cooled to 4°C and filtered using an Aminco ultrafiltration unit with a 10,000 dalton cutoff. The filtered product was lyophilized, and measured portions were added to two collagen suspensions (3 + 4 and 1 + 3 by weight, product added to collagen). These mixtures were then digested by the procedure described above.

RESULTS AND DISCUSSION

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A series of digestions was set up using dura mater tissue from a 36-year-old donor to examine the effect of variables, such as enzyme concentration and collagen concentation. Tissue of this age is no longer growing, and is young enough to be digested readily. Dura mater was chosen because it is a source of quite pure collagen and it is not a physiologically active tissue; possible physical conditioning effects would be negligible. Figure 1 shows three of seventeen digestion curves, run under different digestion conditions. Different digestion conditions have been previously normalized in a first order manner for the concentrations of enzyme and soluble collagen (Mandl *et al.*, 1964). An ability to normalize the kinetic data implies that the volume of base required per ml of solution and the time to a given conversion is an inverse function of the enzyme concentration.

Figure 2 shows the normalized data of Figure 1. While only three curves from the extremes of the concentration ranges are shown for clarity, all digestions normalized to the same curve within limits of experimental error. The first order normalization procedure was also found to be effective for all digestions from several different donor sources. From the variety of conditions used, and the effectiveness of the first order normalization procedure for the digestions, it can be concluded that all digestions are first order in collagen between runs. However, the actual mechanism is more complex.

The data of Figure 1 are plotted in the rate form normalized for collagen and enzyme concentration (dV/Edt) (Figure 3). In this type of plot a first order dependence is shown when a straight line is obtained. Through the normalization procedure, the digestions were found to be first order in collagen and enzyme *between* runs. However, *within* a digestion there is no apparent first order dependence on collagen, since straight lines were not obtained (Figure 3). Further examination of the rate data detected an initial linear portion of the rate curve for digestions done at low enzyme concentrations. Only the first 10 to 15% of the digestion was linear.

Two initial hypotheses were examined to account for deviation from first order kinetics namely that there was either slow enzyme deactivation or product inhibition of digestion. On examining Figures 1 and 2, it can be seen that Run 2 which took 30 minutes for 70% digestion, normalizes with Run 14 which took 4 hours to reach the same point. Thus, there was no significant enzyme deactivation during the time period of the digestion.

The second hypothesis was tested by adding ultrafiltered digestion products to two

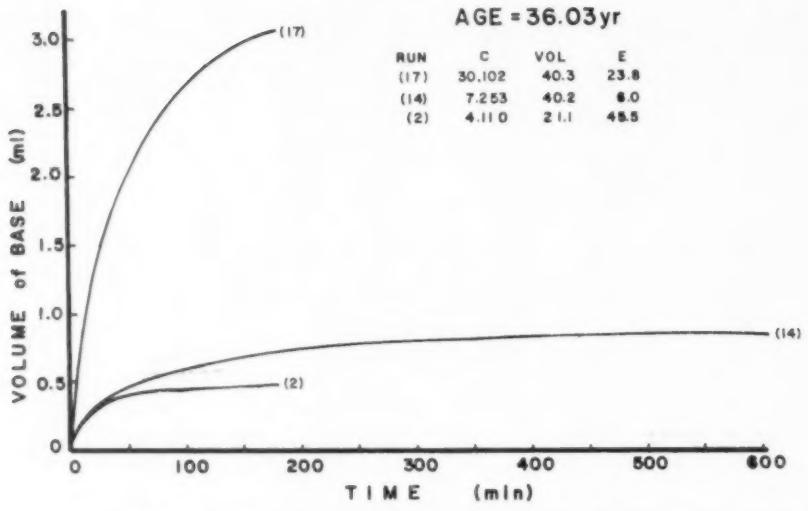

FIG. 1. Volume of base/time digestion curves of dura mater collagen from a 36.03-year-old donor done under various conditions, C = weight of collagen in mg., VOL = volume of digestion mixture in ml., E = enzyme concentration in units/ml.

separate runs and comparing these with the normal runs in the 36-year-old material. These two runs normalized exactly showing that the products did not slow down the reaction. A remaining possibility that explains the deviation from first order kinetics is that there is something inherent in the nature of collagen digestion which determines the shape of the digestion curves.

The apparent effect of aging can be shown in an empirical manner by plotting the normalized time (tE) to reach a given extent of reaction (μl/mg) versus the age. The "tE"

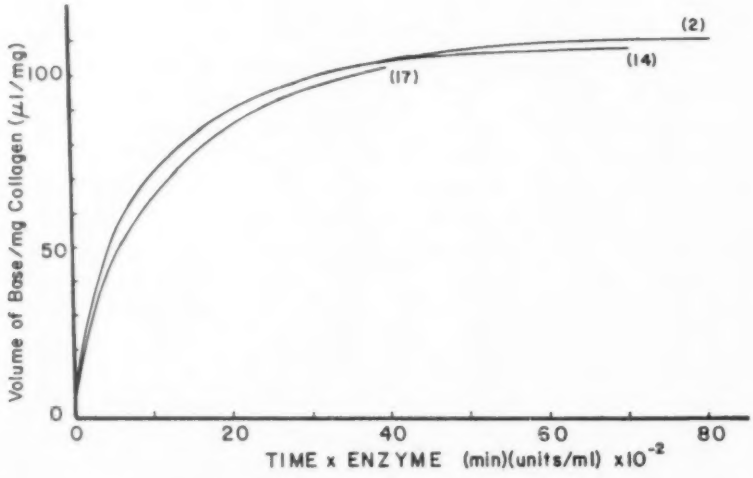

FIG. 2. Normalized volume of base/time curves of dura mater collagen from a 36.03-year-old donor.

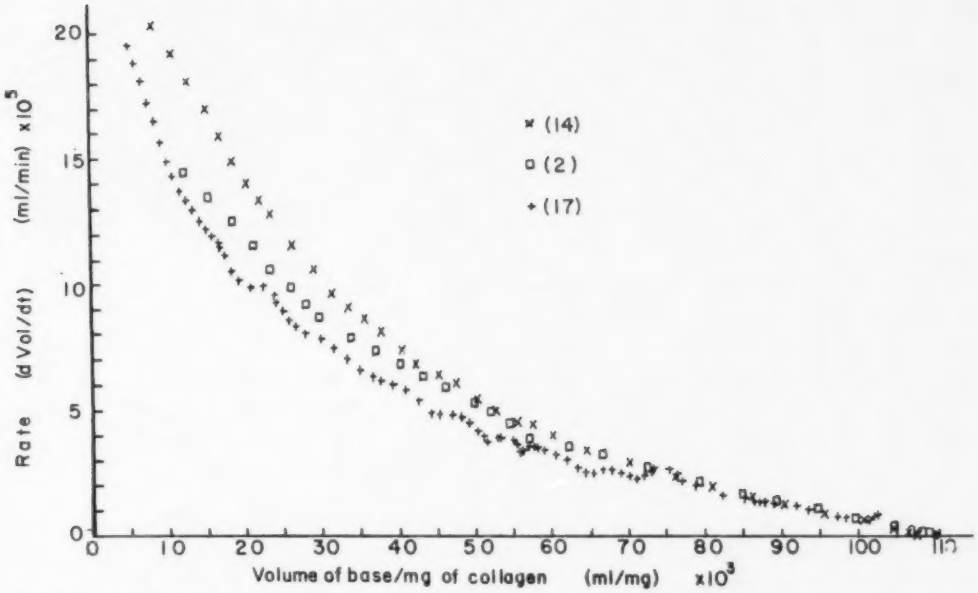

FIG. 3. A plot of the normalized rate of generation of volume base/mg of collagen versus volume of base/mg of collagen for dura mater collagen from a 36.03-year-old donor.

values to reach 24 μl/mg are shown for different sets of runs (Figure 4). Similar curves can be developed for 15 and 35 μl/mg. This extent of reaction is a manifestation of the initial and central portions of the digestion. The effect of age is a linear increase in digestion time from about 20 years (onset of maturity) to about 70 years. The difference between the slopes of the two data sets shows that large differences can exist between different enzyme lots.

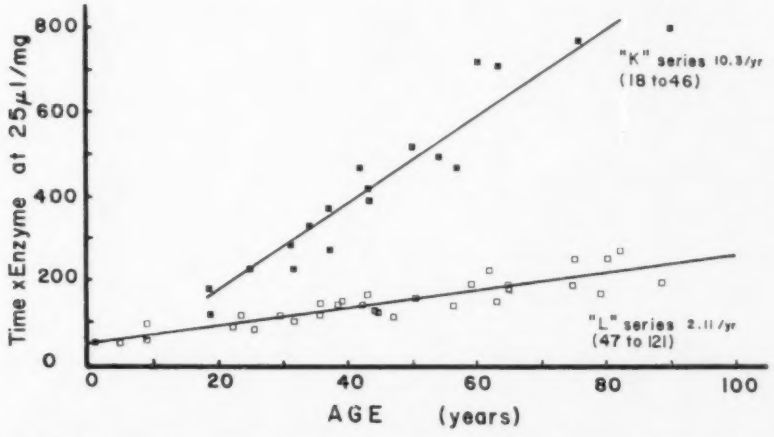

FIG. 4. Plot of the tE values to reach the normalized extent of digestion of 25 μl of base/mg of collagen for various age donors.

The classical Michaelis-Menten enzyme kinetic analysis is first order and only uses initial rates. It has been shown that the digestion of insoluble collagen does not remain first order after the initial stages (Hamlin and Kohn, 1971). This present study also uses the total digestion curve and shows that the apparent reaction order changes *within* a digestion. The volume of base/time data was empirically found to fit equation (1) after the initial stages.

$$t/V = A + Bt \tag{1}$$

Figure 5 shows the digestions of three different-age samples, plotted using equation 1. This equation is a variation of the second order equation, where B is the inverse of the final volume of base (V_∞) and A is a function of the rate constant and V_∞. Although no mechanism is obvious, the effects of age can be seen from changes in the value of A. This equation can be used to follow the age differences. The amount of base needed to restore the selected pH following the addition of enzyme solution (the titration blank) and/or any initial short term, parallel reaction, all affect the value of the A term, while the B term is the summation of these effects on V_∞.

Values of V_∞ are not readily obtainable even from equation 1, since V includes the unknown blank titration as well as the first order initial digestion contribution, even if the digestion is run to completion. The use of integral equations such as (1) is not particularly desirable since B, the slope, cannot be easily separated into its components. In the derivative form, a treatment is possible which can extract values for the initial first order

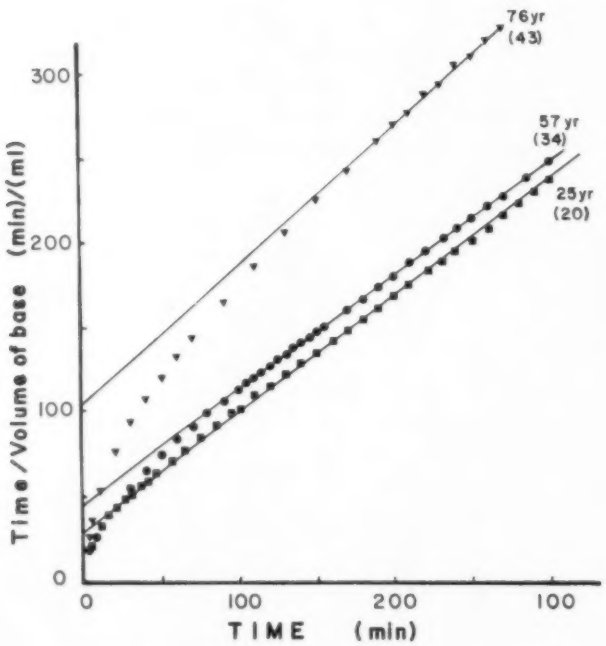

FIG. 5. Plot of volume of base/time digestion data using eq. 1 for three samples of diaphragm tendon collagen of various ages.

reaction and the secondary, more complex, reaction. This is discussed in the next paper. However, much of the reaction is second order, at least in samples from 25 to 57 years of age.

There are at least two parallel reactions occurring during the digestion; the initial one seems to be a simple first order reaction with respect to collagen. The normalization procedure also shows that the total digestion is first order in collagen and enzyme concentration. The digestion is always first order with respect to collagenase, otherwise the non-linear portions in Figure 2 would not coincide within experimental error. Since the non-linear portion of the rate versus volume of base curves were fitted to a second order equation in Figure 3, the digestion rate is a function both of the collagen concentration and the fraction of remaining collagen. These kinetics are not found in reactions of simple molecules. However, collagen is a complex structure with crosslinks and non-digestible portions and its digestion need not necessarily follow simple laws.

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HUMAN COLLAGEN DIGESTION: II. THEORETICAL MODEL AND APPLICATION TO DATA

JAMES S. HOLTON*, MORTON H. LITT, ROBERT R. KOHN, and CLIVE R. HAMLIN†

Department of Macromolecular Science (J.S.H. and M.H.L.), and Institute of Pathology, School of Medicine (R.R.K. and C.R.H.), Case Western Reserve University, Cleveland, OH 44106, U.S.A.

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Abstract—A kinetic mechanism for the digestion of collagen by bacterial collagenase has been proposed which is consistent with experimental data. Arguments are presented justifying the application of a homogeneous approach to the digestion of insoluble collagen. The mechanism takes account of increased kinetic blockages with aging and enzyme complexity. It is shown to be applicable to collagen from various tissues, including relatively impure preparations. However, the mechanism is not applicable to collagen from the immature period of the lifespan.

Kinetic blockages are shown to increase three- to four-fold from age 20 to 70 years, within the main helical region of the collagen molecule. There is no increase of kinetic blockages in the N-terminal region of the molecule over this age range.

INTRODUCTION

THE PRECEDING PAPER presented the criteria for the mechanism of collagenase digestion of collagen of various ages (Holton *et al.*, 1983). These are:

1. The digestion is a simple first order reaction with respect to enzyme.
2. The enzyme activity is not inhibited by the digestion products, nor does it autodeactivate during the test time.
3. There are at least two collagen dependent reactions with the enzyme. The digestion therefore is a complex first order reaction with respect to collagen.
4. The rate constant of the major portion of the reaction seems to be a function both of the age of the collagen and collagen conversion.

These criteria are used to generate a mechanism which is applied to various sets of data.

The digestion model is developed using the α1 (I) strand of collagen. The N-terminus is very susceptible to collagenase attack, compared to attack at the center of the chain (Stark and Kuhn, 1968a, b). This difference in digestion susceptibility allows the collagen strand to be considered as two separate sections. A model for the collagen α1 (I) molecule which emphasizes the qualities for the digestion criteria is shown in Figure 1. Starting from the N-terminus there is a section, labelled C_e, which can be rapidly digested by an exopeptidase. The remainder of the chain, labelled C_c, can be split by an endopeptidase. The C_c

*Present Address: ARCO Chemical Company, 3801 West Chester Pike, Newton Square, PA 19073, U.S.A.
†To whom reprint requests should be addressed.

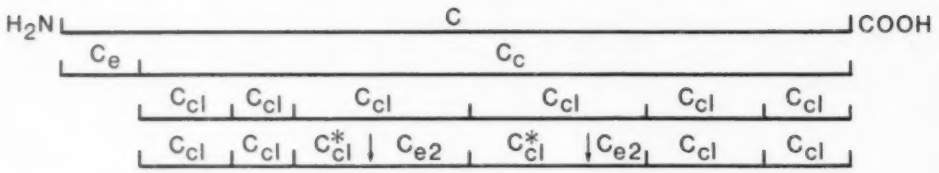

FIG. 1. Schematic of a collagen strand before and during digestion using proposed digestion mechanism.

section is presumed to contain additional sub-sections interspersed by covalent cross-linked sites, or other stabilizing components that act as kinetic blockages. The kinetic blockages divide the C_c section into small subsections (C_{cl}) subject to initial cleavage by collagenase acting as an endopeptidase. These subsections are considered as kinetically independent reactive segments. As previously noted, the reactivity of collagenase toward the various cleavable amino acid sequences varies (Holton *et al.*, 1983). Consequently the rate of cleavage at any site is considered to be an average value as many strands are being digested at any one time.

Kinetically, the digestion consists of two reactions. The first is the attack at the N-terminus of the helical portion of the strand (C_e) by enzyme in a simple first order mechanism. This part of the digestion is the cleavage of C_e to final product, containing no cleavage sites, until the first kinetic blockage is reached.

The second and major reaction is a two step consecutive first order attack on the C_{cl} segments of the collagen strand. The first step is the complexing of the enzyme on the C_{cl} segment and cleavage of the C_{cl} segment at a random cleavage site. This cleavage forms two new ends on the strand, labelled C_{e2} (with an N terminus) and C^*_{cl} (with a C terminus). The C^*_{cl} strand end is essentially as unreactive as the initial C_{cl} segment, while the C_{e2} is easily and rapidly cleaved to final product until a kinetic blockage is reached.

The original C_{cl} is thus replaced by a similar but shorter C^*_{cl}. Since the attack on C_{cl} or C^*_{cl} is at random, the average length of the C^*_{cl} and C_{e2} segments formed is one-half of the C_{cl} or previous C^*_{cl} segment cleaved. Therefore, the average length of the C_{cl} or C^*_{cl} segments and the amount of product formed per cleavage, that is length of the C_{e2} segment, decreases with conversion. This implies that the rate determining step of the digestion of C_c is the cleavage of its C_{cl} subsegments. The number of C_{cl} plus C^*_{cl} subsegments remains constant until the last stages of digestion.

The digestion is monitored by titrating the acid groups formed in the cleavage of collagen by collagenase. The rate at which base is used, or product is formed, is dependent both on the rate of cleavage of the C_{cl} segments and their average size at any time.

Kinetic equation development

The mechanism of the first reaction, digestion of C_e, is apparently a simple first order reaction as shown in equation 1 with the rate of product formation from C_e segment being given in equation 2.

$$C_e + E \xrightarrow{k_1} P_e + E \tag{1}$$

$$dP_e/dt = -dC_e/dt = k_1(E)(C_e) \tag{2}$$

The term C_e represents the total number of cleavage sites in the original N terminal segment. Other possible, more complex mechanisms exist; however, their resulting equations reduce to equation 2.

The second and major reaction of the digestion is the attack of the enzyme on the portion of the strand helix containing the kinetic blockages (C_c). This reaction is proposed to occur as a two step consecutive first order reaction with respect to collagen. The equations describing the mechanism are

$$C_{c1} + E \underset{}{\overset{K}{\rightleftharpoons}} (EC_{c1}) \overset{k_2}{\rightarrow} C^*_{c1} + E + C_{e2} \tag{3a}$$

$$C_{e2} + E \overset{k_3}{\rightarrow} P + E \tag{3b}$$

Equation 3a represents the proposed rate determining step, the complexing of the strand segment C_{c1} with enzyme, and its subsequent cleavage into two subsections, C^*_{c1} and C_{e2}. In equation 3b, the new C_{e2} end forms product by the mechanism discussed for C_e.

From the proposed mechanism, there is assumed to be no kinetic difference between an original strand segment C_{c1} and a segment formed by cleavage, C^*_{c1}. During the digestion, the number of segments remains constant at the initial number until the last stages of the digestion, but the average segment size will decrease. Furthermore, the initial average size of a strand segment C_{c1} will decrease as the number of kinetic blockages increases. So, as the total number of final product units liberated per initial cleavage depends on the age of the collagen and the amount of collagen remaining, equation 3a can be rewritten as

$$-dC_{c1}/dt = dP/dt = k_2(N)\,[E]\,[C_{c1}] \tag{4}$$

where N is the number of peptide fragments liberated by the digestion of the N-terminal section produced from the initial cleavage of a C_{c1} subsection. Equation 4 will not be valid in the initial stages of the reaction while the original N-terminal end, C_e, is being digested, but it should hold afterwards.

The number of fragments obtained from the cleavage of a C_{c1} segment is a function of the initial distribution of segment lengths and their degree of reaction or conversion (C_{c1}/C'_{c1}). The average amount of acid product liberated per internal cleavage, N, can be defined as the total number of cleavage sites (S) in strand C_c divided by twice the number of kinetic blockages (2X) (because on the average a cleavage will allow half of a C_{c1} to be digested) times the fraction of remaining collagen. Therefore, (N) can be redefined as:

$$N = (S/2X)\,(C_{c1}/C'_{c1}) \tag{5}$$

Substituting this into equation 4, we obtain equation 6.

$$dP/dt = -dC_{c1}/dt = k_2(S/2X)\,(E)\,(C_{c1}/C'_{c1})C_{c1} \tag{6}$$

Although the mechanism is first order in collagen, the final equation, equation 6, is second order in collagen. This is why a second order equation could be successfully applied to the data obtained from the digestion (Holton *et al.*, 1983). This type of apparent second order

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result has also been observed in a first order consecutive depolymerization mechanism (Boyd, 1970).

Equation 6 can be integrated and solved for C_{c1} using

$$-\int_{C'_{c1}}^{C_{c1}} dC_{c1}/C_{c1}^2 = k_2D(E)/C'_{c1}\int_{o}^{t} dt \tag{7a}$$

where D = S/2X.

Therefore

$$1/C_{c1} - 1/C'_{c1} = k_2D(E)t/C'_{c1} \tag{7b}$$

which rearranges to

$$C_{c1} = C'_{c1}/(1 + k_2D(E)t) \tag{7c}$$

Putting equation 7c into equation 6 gives

$$dP/dt = k_2D(E)C'_{c1}/(1 + k_2D(E)t)^2 \tag{8}$$

The quantity C'_{c1} is not directly obtainable since the number of segments (and their length) are determined by the initial kinetic blockages. However, the number of cleavage sites for all of the C_{c1} strands is fixed by the amino acid sequence. This means that the amount of product formed in complete digestion is fixed per unit mass of collagen. Therefore we have normalized all data by using product per mg of collagen rather than total product.

The reaction of the original N terminus (C_e) is faster than the digestion of C_c and comprises only 10% of the total digestion. Therefore, by the time a conversion of 15 to 25% is reached (the two reactions are parallel), the observed rate of digestion will be due only to the C_c segments.

The term C_{c1} in the equations represents the concentration of cleavable sites in the remaining segments and is equal to C_c, the total concentration of cleavable sites remaining. This equality allows the replacement of C_{c1} by C_c in equation 6 and equation 8 to give:

$$dP/dt = k_2D(E)C_c^2/C'_c \tag{9}$$

$$dP/dt = k_2D(E)C'_c/(1 + k_2D(E)t)^2 \tag{10}$$

These equations describe the formation of product during the latter part of the digestion. In order to analyze the data, which has been put in terms of volume of base added per mg of collagen and time, P, C_c, C'_c, C'_e must be defined in terms of normalized volume of base. Table 1 lists and defines the equalities. [K is a proportionality constant, converting milligrams of collagen or product to volume of base. It cancels out of all equations used, and is not determined. It is a function of the isoelectric pH of the products in solu-

TABLE 1. EQUALITIES USED TO CONVERT EQUATIONS 9 AND 10 TO EQUATIONS 11 AND 12.

Collagen	*Volume of Base/mg Collagen*	
C'	$K V_\infty$	Total number of cleavage sites per mg collagen
C'_e	$K V_{1\infty}$	Total cleavage sites in the initial N terminus segment per mg collagen
C'_c	$K(V_\infty - V_{1\infty})$	Total cleavage sites in the main helical segment per mg collagen
C_c	$K(V_\infty - V)$	Cleavage sites at any $t(o < t < \infty)$ in the main helical segment after C_e has been digested
P	K V	Product per mg collagen formed by digestion
K		The constant to convert amount of collagen to volume of base (not determined)

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tion and the set pH of the system.] After substitution of these equalities into equations 9 and 10, they were rearranged to a normalized linear form giving:

$$(dV/Edt)^{1/2} = (k_2D/(V_\infty - V_{1\infty}))^{1/2}V_\infty - (k_2D/(V_\infty - V_{1\infty}))^{1/2}V \quad (11)$$

$$(dV/Edt)^{-1/2} = (k_2D)^{-1/2}(V_\infty - V_{1\infty})^{-1/2} + (k_2D/(V_\infty - V_{1\infty}))^{1/2}Et \quad (12)$$

There is one significant advantage to using equations in the derivative form rather than the integrated form. The unknowns, V_∞ and $V_{1\infty}$, k_2D, etc., can be derived directly from the plots of equations 11 and 12. In the integral form, values for V_∞ or $V_{1\infty}$ must be postulated in order to find the other constants. As the titration curves are quite accurate, the rates were easily found by fitting seven equally spaced points to a parabola using a least squares fitting routine and then calculating the rate at the center point. This method gives a value for dV/Edt at a given time and V.

The ratio of the intercept to the slope of equation 11 is V_∞, the volume of base needed for complete digestion. The ratio and product of the slope and intercept of equation 12 allow the evaluation of k_2D, the apparent initial C_c digestion rate constant, and

$V_\infty - V_{1\infty}$, the volume of base for complete digestion of the C_c strand segments. The absolute values of the slopes of the two equations are identical, giving an internal check when the equations are applied to the data.

The limitations on the conditions where equations 11 and 12 are valid are: 1) The rate of product formation must be a result only of the reaction described in equation 3, that is the digestion of the N terminus must be completed, and 2) the distribution of strand segment lengths must be essentially random; if not, equation 5 will be invalid because N will not be a linear function of conversion.

An additional assumption is that cleavable segment sites adjacent to a crosslink may digest very slowly or not at all. Under conditions where the number of cleavage sites per strand segment is small, the loss of these potential cleavage sites cannot be neglected. Therefore as the number of kinetic blockages present in the collagen strand increases or the initial average segment length decreases, the digestion might show a smaller total number of cleavages (a lower V_∞ value) than under low kinetic blockage conditions.

There are approximately 160 possible cleavage sites and assuming seven kinetic blockages in the C_c segment, the average subsection would be approximately 23 cleavage sites long. For this situation, an average remaining sequence length of two corresponds to a conversion of 92%. If there were 20 kinetic blockages, about three times the former level, or 8 cleavage sites per average subsection, an average length of two sites could be reached at a conversion of 75%. These values would correspond to a lower V_∞ if enzyme attack adjacent to a kinetic blockage is hindered. V_∞ would decrease as the age of the subject increases, which is found. Also, the equations are for infinitely divisable systems, not for the real system containing a limited number of cleavage sites per subsection. When some subsections become so short that they can no longer be cleaved, the basic kinetic conditions are no longer fulfilled. Therefore, equations 11 and 12 are valid only from the time they become linear to a conversion level where the average subsection length is small.

MATERIALS AND METHODS

Digestion data were obtained from results previously described, or were supplied by Hamlin and Kohn (1976).

RESULTS

The rate data from a single digestion were applied to equations 11 and 12 respectively (Figures 2 and 3). The plots are linear over 75% of the volume titrated and 90% of the time of the digestion for this run. Runs on older samples are linear over about 60% of the digestion time. The slopes and resultant parameters (V_∞, $V_\infty - V_{1\infty}$, k_2D), were calculated using linear regression on sets of data points. The values of the parameters were chosen by minimizing the difference between the slopes of equations 11 and 12 for each data set by adjusting the number of data points used (>40) to maximize the regression coefficient.

One data group consisted of digestions of collagen from the 36.03-year-old donor run under various conditions. The differences in slope from equations 11 and 12 ranged from 0.06 to 5.4% for all the digestions, with most around 1–2%. Table 2 lists the average values for k_2D, V_∞, $V_\infty - V_{1\infty}$, and $V_{1\infty}$ for the different parameters of volume, enzyme concentration and mass of collagen. There were no trends for any of these parameters. Furthermore, matched pairs of runs on samples, both with and without sonication prior to digestion, showed no significant difference between the kinetic values, although the

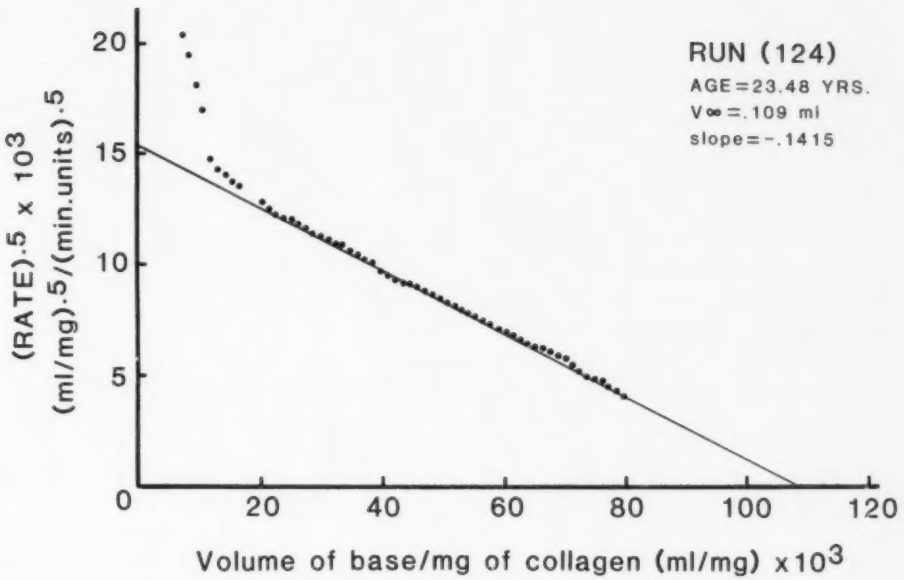

FIG. 2. Application of equation 11 to normalized digestion data from collagen of a 23.48-year-old donor.

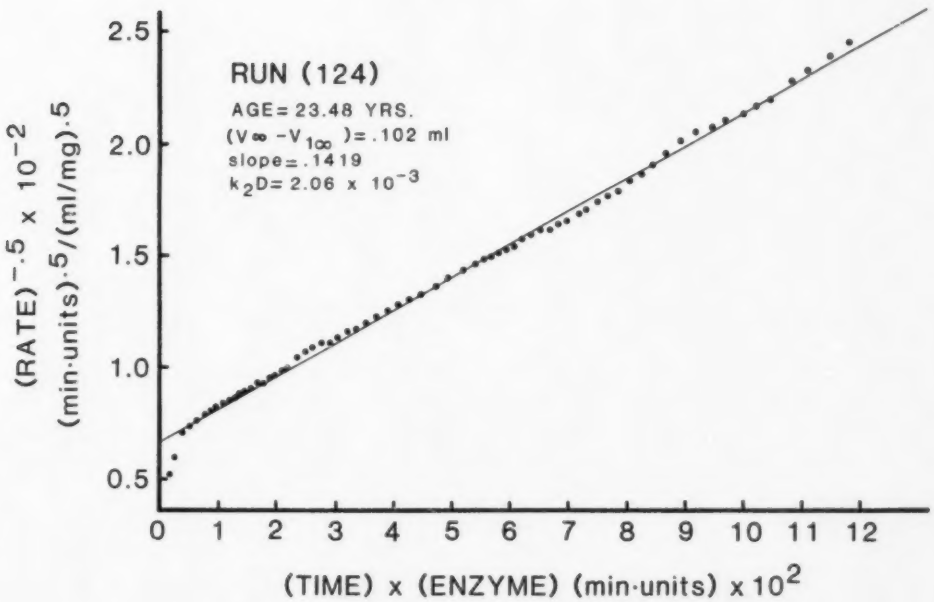

FIG. 3. Application of equation 12 to data used in Fig. 2.

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TABLE 2. VARIATION OF DIGESTION PARAMETERS OF COLLAGEN FROM A 36.03-YEAR-OLD DONOR AS CALCULATED FROM EQUATIONS 11 AND 12.

Number of Runs	*~ Vol. (ml)*	*~ Mass (mg)*	*[E] (units/ml)*	$k_2D \times 10^3$	V_∞ *μl/mg*	$V_\infty - V_{1\infty}$ *μl/mg*	$V_{1\infty}$ *μl/mg*
21	15–40	4–30	3–45	1.09 ± .15	118.0 ± 9.7	105.4 ± 8.5	12.6 ± 2.6

data from sonicated samples were more reproducible. These observations provide partial justification for the application of a homogeneous kinetic approach to the digestion of insoluble collagenous tissue fragments.

The data from immature samples do not follow equations 11 and 12. There is curvature for most of the digestion due to non-random distribution of the initial average segment lengths. A linear portion of the curve at the end of the digestion indicates the remaining segment fragments have become random due to the random attack of the enzyme, and equation 11 does apply to those data. However, any values of k_2D obtained from this portion of the curve do not reflect the initial segment size distribution.

The apparent value of k_1 was calculated from the slope of the initial linear portion of the rate *versus* volume-of-base curves (Figure 2). The value of k_1, determined at low enzyme concentrations with collagen from the 36.03-year-old donor was $10.65 \pm 2.7 \times 10^{-3}$. There is more error associated with this datum than for k_2D because the data used are located in the portion of the digestion which is least accurately measured, and because the second, two stage consecutive reaction, is also proceeding and its rate of product formation affects the values obtained for k_1 and $V_{1\infty}$. Therefore, the value of $V_{1\infty}$, 29.0 ± 3.4 μl/mg, is a summation of titrated product from the first reaction and a portion of the second reaction. This can be seen by comparing this $V_{1\infty}$ value with that shown in Table 2.

When the mechanism described in equation 3 is used to describe the effect of age on collagen samples, the change is contained in the value of k_2D, determined from equation 12, which can be followed by use of equation 5. In this model the cleavage rate (k_2) for crosslinked collagen remains approximately constant as does the number of cleavage sites, C_c, with increasing age. The parameter which changes is X, the number of kinetic blockages. A plot of the reciprocal of k_2D versus age would show directly how X changes with age. These values are plotted for two different sets of data in which the enzyme lots were different (Figure 4). The plotted values are averages of each age and the error bars represent the standard deviations of the $(k_2D)^{-1}$ data when they were larger than the symbols used.

Heart muscle collagen of a lower purity (30–40% collagen) than the dura mater and diaphragm tendon (95%) was digested using the protocol described above (Zwolinsky *et al.*, 1976). When all data were normalized per mg of collagen based on the hydroxyproline analysis, it was evident that digestion stopped when the collagen had been digested even though other protein was still present.

The heart muscle collagen data were analyzed using equations 11 and 12. Normal values obtained were for V_∞, 115 to 100 μl/mg depending on the run, with k_2D values of 3×10^{-3} to $.7 \times 10^{-3}$ depending on the age of the sample. These values are comparable to the values obtained using dura collagen of the same age.

The value of V_∞ appears to decrease with age (Figure 5). There is marked scatter, which

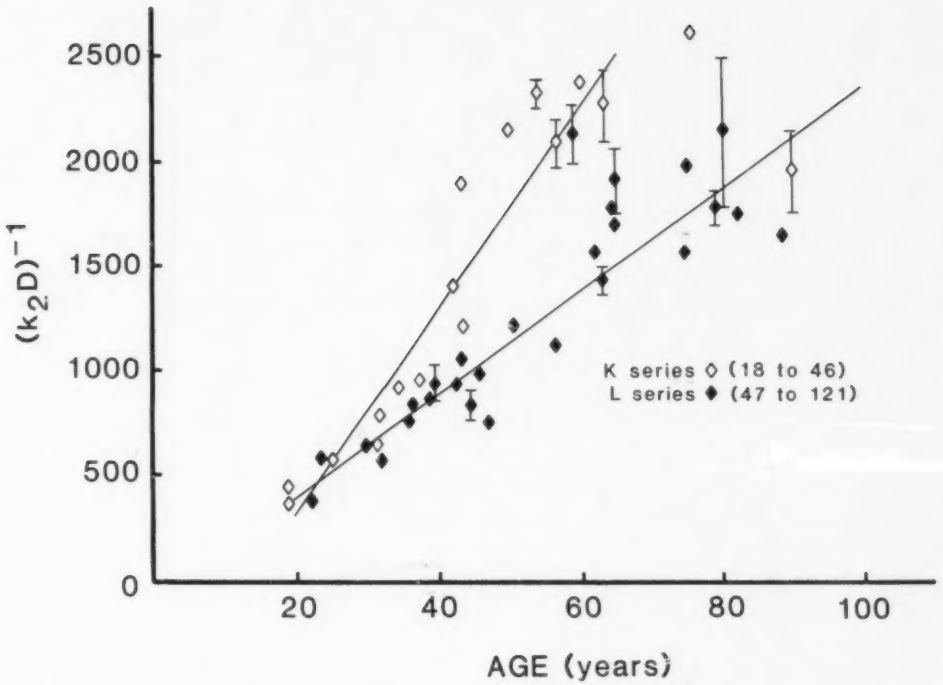

FIG. 4. Plot of the reciprocal of the major reaction, apparent rate constant (k_2D) versus the collagen donor age.

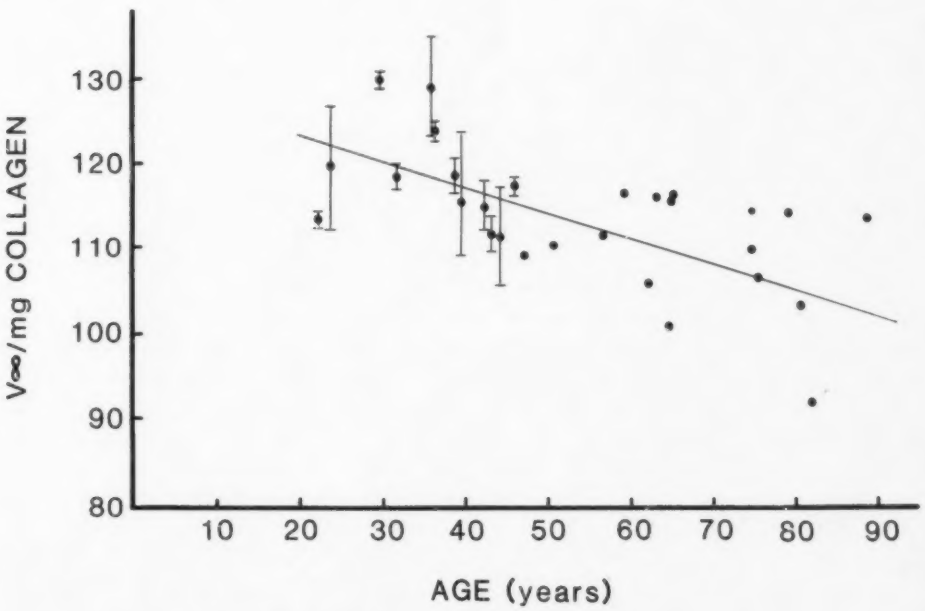

FIG. 5. Plot of V_∞/mg collagen versus the collagen donor age.

increases with increasing age, but the trend is clear. The average V_∞ drops from about 123 μl/mg at 20 years to about 105 μl/mg at 80 years.

DISCUSSION

The application of a homogeneous kinetic approach to the digestion of insoluble, collagenous tissue fragments is not obviously justifiable. However, kinetic models which depend on heterogeneous attack, surface area alone or combined with diffusion do not fit the actual data. Consider the attack on a cylindrical fibril occurring from the outside in, a surface area model. This model leads to a linear dependence of the digestion on the surface area of the cylindrical fibril or a square root dependence of digestion on remaining collagen. Alternatively, in a diffusion model, the enzyme cleaves surface linkages, allowing the outside part of the fibril to swell. Enzyme diffuses in before all the surface material is digested, cleaving and swelling interior parts. This leads to a sigmoidal digestion curve as a larger fraction of the collagen is attacked later than initially. Neither model yields a second order curve.

We hypothesize that the collagen fragments swell enough at the microfibrillar level to permit enzyme penetration in a very short time compared to the digestion rate. All monomeric collagen components are subject to attack. Enzyme may be able to penetrate the microfibril more readily once the N-terminal portion is removed. The second order dependence of digestion rate comes about because the enzyme is complex, functioning as both an exopeptidase (fast reaction) and an endopeptidase (slow reaction). Slow cleavage of an internal segment generates an amino-terminated chain, which then digests very rapidly until a kinetic blockage is reached. As digestion proceeds, the digestible sequence generated per internal cleavage gets shorter. Since the collagen remaining to participate in the internal cleavage reaction also decreases with conversion, the rate at which acid groups are liberated becomes second order in collagen.

Experimentally derived data are consistent with the proposed mechanism. Data from a single digestion, when applied to equations 11 and 12 yield essentially identical slopes, providing an internal check between equations. The digestion parameters are independent of the enzyme concentrations, mass of collagen or the total volume during digestion (Table 2). The digestion rate is relatively independent of the state of aggregation as shown by the sonication experiments. Equations 11 and 12 are applicable to the digestion of partially purified collagen and yield similar digestion parameters when dura collagen is digested. This shows we are observing only the attack of collagenase on collagen, with no contribution from trace amounts of other proteolytic enzymes in the collagenase preparation. Differences between enzyme lots (Figure 4) are probably due to differences in the relative proportions of collagenase subfractions and not to contaminating proteases.

Further support for the proposed mechanism comes from calculating the apparent values of k_1. A value of k_1 (exopeptidase) of about 10×10^{-3} l/m sec. compared to k_2D of 1.1×10^{-3} l/m sec. was obtained on a 36-year-old specimen. This predicts that by the time 20 to 30 percent of C_c is removed, the digestion of the N-terminus should be essentially complete. As can be seen in Figure 2, this is where the plot becomes truly second order.

The proposed mechanism was used to examine the effect of age on collagen. The cleavage rates k_1 and k_2 remain essentially constant, as does the number of cleavage sites, C_c, with increasing age. The number of kinetic blockages, X, is the parameter which changes (equation 5). The plot of $(k_2D)^{-1}$ versus age showed directly how X changed with

age (Figure 4). The number of kinetic blockages at age 70 years are about three to four times those present at age 20, assuming the molecular weight distribution of the collagen monomers remains unchanged. At 70 to 80 years of age, the $(k_2D)^{-1}$ values tend to level off. There may be then no more sites to create additional kinetic blockages.

A second effect of age on collagen is shown by the apparent decrease in the volume of base needed for complete digestion (V_∞) with age (Figure 5). About 15 percent of the collagen seems to become inaccessible to the enzyme between 20 and 70 years of age, presumably due to increased kinetic blockages. A kinetic blockage may protect two or even more cleavage sites depending on its location. Consequently the loss of 15 percent of digestible residues could be due to up to ten additional kinetic blockages formed between ages 20 and 70 years.

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